CLXIX. THE ACTIVATION OF FEMALE SEX HORMONES

III. MONO-ESTERS OF α-OESTRADIOL

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Two series of mono-esters are derived from α-oestradiol according to whether the acid radical is in the 3- or 17-position.

Up to the present only one mono-ester has been thoroughly investigated, viz. oestradiol-3-benzoate prepared by Schwenk & Hildebrandt [1933]. According to Kaufmann [1933; see also Schoeller *et al.* 1935] it has a protracted effect in comparison with the free follicular hormone. In the previous paper of this series [Miescher *et al.* 1938, 2] we showed that as regards duration of effect and output of activity many di-esters of oestradiol are much better than the 3-benzoate. The same applies also to the series of the 3- and 17-mono-esters. The investigation of the esters was made by determining their threshold value and duration of effect in the oestrus test as well as their action in the uterus growth test using rats in the same manner as described previously. Again the esters were injected subcutaneously in oily solution. 1275 animals were used for these experiments.

The mono-esters of α-oestradiol investigated by us are shown in Table I.

Table I

Oestradiol mono-esters	M.P.* °C.	Oestrus test (rat): Threshold value S_r rat oestradiol $=0{\cdot}4\gamma$	Oestrus test (rat): Threshold value S_o based on oestradiol $=0{\cdot}1\gamma$
3-Acetate	136·5–137·5	0·4	0·1
3-Propionate	125·0–126·0	0·4	0·1
3-*n*-Butyrate	98·0– 99·0	0·4	0·1
3-*n*-Valerate	Approx. 58·0– 60·0	0·4	0·1
3-*n*-Hexanoate	Approx. 46·0– 51·0	0·4	0·1
3-*n*-Octanoate	Approx. 48·0– 53·0	0·6	0·2
3-*n*-Decanoate	59·0– 60·0	2·0	0·5
3-Palmitate	70·0– 71·0	7·0	1·8
3-Stearate	78·0– 78·5	7·0	1·8
3-Benzoate	193·0–194·0	0·75	0·2
17-Acetate	215·0–216·5	0·5	0·1
17-Propionate	199·0–200·0	0·5	0·1
17-*n*-Butyrate	166·5–167·0	0·5	0·1
17-*iso*Butyrate	183·0–183·5	0·5	0·1
17-*n*-Valerate	144·0–145·0	0·5	0·1
17-*n*-Hexanoate	128·5–129·0	0·7	0·2
17-*n*-Octanoate	117·5–118·0	0·8	0·2
17-*n*-Decanoate	112·0–112·5	3·5	0·9
17-Benzoate	93·0– 94·0	3·0	0·8
17-Methylcarbonate	216·5–218·0	0·6	0·2
17-Ethylcarbonate	171·0–172·0	0·6	0·2
3:17-Diethylcarbonate	137·5–138·0	1·5	0·4

* The melting-points are taken with a short thermometer.

Apart from the 3-benzoate only the two mono-acetates have been mentioned in the literature. The preparation of a series of mono-esters has already been reported by us elsewhere [Miescher & Scholz, 1937, 1, 2]. A description of some further mono-esters is appended.

Oestradiol-3-n-*valerate.* 630 mg. oestrone-*n*-valerate were dissolved in 95 ml. ethyl acetate (containing no alcohol) and mixed with 350 mg. platinum oxide (Adams). The whole was shaken at room temperature under a pressure of hydrogen of 4·3 atm. for 20 hr. After filtering off the platinum and evaporation of the solvent under diminished pressure the remaining oil was purified for analysis by two distillations at 0·0004 mm. (bath temperature 180–190°). (Found: C, 77·61; H, 9·01%. $C_{23}H_{32}O_3$ requires C, 77·47; H, 9·06%.) The oil can be crystallized from alcohol-water but the crystals contain solvent. M.P. about 58–60°.

Oestradiol-3-n-*hexanoate.* A solution of 428 mg. oestrone-*n*-hexanoate in 65 ml. alcohol-free ethyl acetate was mixed with 220 mg. platinum oxide and reduced as above. The working up was the same. After distilling 3 times at 0·0008 mm. (bath temperature 180–190°) a colourless oil was obtained which could be crystallized from alcohol-water; however, the crystals contain solvent. The M.P. is about 46–51°. (For the oil, found: C, 77·81; H, 9·20%. $C_{24}H_{34}O_3$ requires C, 77·78; H, 9·26%.)

Oestradiol-3-n-*octanoate.* In the same manner oestrone-*n*-octanoate, M.P. 69·8–70·2°, was reduced by means of platinum oxide in ethyl acetate and an oily residue obtained which was distilled 3 times at 0·0004 mm. (bath temperature 180°). The oil was dissolved in alcohol-water and after standing, crystals were formed, containing solvent. M.P. about 48–53°. (For the oil, found: C, 78·45; H, 9·46%. $C_{26}H_{38}O_3$ requires C, 78·33; H, 9·62%.)

Oestradiol-3-n-*decanoate.* The oestrone-*n*-decanoate was reduced in the manner described above. After filtering and evaporation of the ethyl acetate under diminished pressure an oil remained which after recrystallizing several times from methanol gave fine needles with constant M.P. 59–60°. (Found: C, 77·05; H, 9·97%. $C_{28}H_{42}O_3$, 0·5 H_2O requires C, 77·18; H, 9·95%.)

Oestradiol-17-n-*hexanoate.* 960 mg. oestradiol-di-*n*-hexanoate (an oil) were mixed with 60 ml. of 0·5% K_2CO_3 in 95% methyl alcohol and stirred for about 3 hr. at room temperature during which time solution occurred. The solution was then slightly acidified and by slowly adding water crystals were precipitated. After filtering off and thoroughly washing first with water and then with diluted Na_2CO_3 and again with water, almost pure oestradiol-17-*n*-hexanoate was obtained and recrystallized several times from methanol-water. M.P. 128·5–129°. (Found: C, 77·67; H, 9·29%. $C_{24}H_{34}O_3$ requires C, 77·78; H, 9·26%.)

Oestradiol-17-n-*octanoate.* Partial saponification of oestradiol-di-*n*-octanoate (an oil) with 0·5% K_2CO_3 in 95% methyl alcohol was carried out as above. The working up was the same. After purifying by recrystallization several times from aqueous methanol the crystals had M.P. 117·5–118°. (Found: C, 78·37; H, 9·85%. $C_{26}H_{38}O_3$ requires C, 78·33; H, 9·62%.)

I. *Assay in the oestrus test*

3-Mono-esters. As will be seen from Table I the threshold value in the oestrus test, determined on the rat, is the same from the 3-acetate up to the 3-hexanoate, and is the same as that of free oestradiol ($S_r = 0{\cdot}4\gamma$). From the 3-octanoate it increases but does not exceed 7γ even with the palmitate and stearate. As regards duration of oestrus (see Fig. 1) with doses of 50γ only a slight increase of the duration of effect is to be seen up to the hexanoate (from 4 to 8 days). With the 3-octanoate the effect increases suddenly up to 52 days and attains with the 3-stearate a maximum of 80 days[1]. On the other hand, the duration of effect with similar doses of the 3-benzoate is only 15 days. With the lower esters up to the 3-octanoate, oestrus appears on the 4th day, that is, one day later than with oestradiol. With the higher esters oestrus appears on the 5th day.

17-Mono-esters. In comparison with the 3-mono-esters the threshold value of this series is slightly increased. The most pronounced difference is observed with

[1] According to a private communication from Prof. Schoeller, Berlin, the 3-palmitate in a previous experiment on a monkey also showed a long-lasting effect.

the 17-benzoate (3γ) compared with the 3-benzoate ($0{\cdot}75\gamma$). As regards the duration of oestrus (Fig. 2) the 17-mono-esters exceed the corresponding 3-mono-esters considerably. Using 50γ the 17-acetate and 17-propionate show the same duration of effect as the 3-benzoate (15 days). With the higher esters it increases

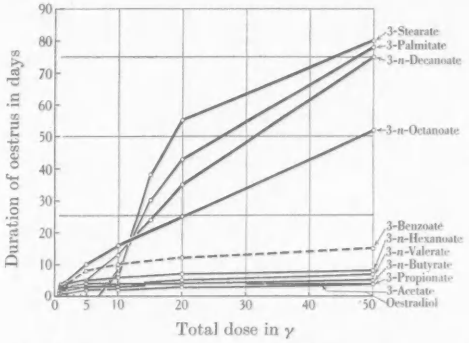

Fig. 1. The effects of aliphatic 3-mono-esters of oestradiol in comparison with oestradiol and its 3-benzoate on castrated rats in the oestrus test, using various doses.

rapidly. The 17-octanoate is of particular interest as, especially with comparatively small doses, it exhibits the most intense action of all esters hitherto investigated. With 5γ the oestrus lasts 30 days, with 20γ 60 days and with 50γ 86 days. With the higher esters (decanoate) the effect appears to diminish. The duration of effect of 17-benzoate (60 days) is 4 times that of the 3-benzoate (15 days). With the 17-mono-esters onset of oestrus is rather more rapid than with the 3-mono-esters. All the 17-mono-esters investigated show their full effect on the 4th day.

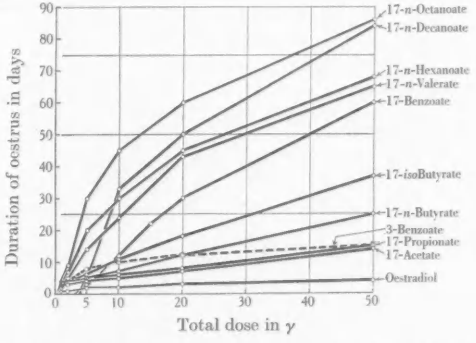

Fig. 2. The effects of 17-mono-esters of oestradiol in comparison with oestradiol and its 3-benzoate on castrated rats in the oestrus test, using various doses.

In Fig. 3 the threshold values (S_r) and duration of oestrus (T_{oe}) of the 3- and 17-mono-esters (with doses of 50γ) are given for comparison. The superiority of the 17-esters over the corresponding 3-esters as regards duration of oestrus is clear. A comparison of this figure with Fig. 2 in the previous paper [1938, 2] is

very instructive. It will be seen that with doses of 50 γ none of the 3- and 17-mono-esters studied is equal to the most active di-esters as regards duration of effect.

In addition to these 17-mono-esters we examined also two 17-monocarbonic esters as well as one 3:17-di-ester of oestradiol using a dose of 50 γ. The duration of oestrus is with the 17-methylcarbonate 14 days, with the 17-ethylcarbonate 25 days and with the diethylcarbonate of oestradiol 40 days.

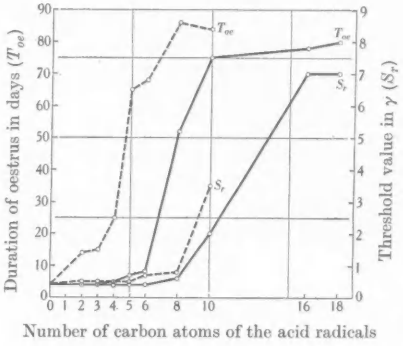

Fig. 3. Comparison of the aliphatic oestradiol -3-mono-esters (———) and -17-mono-esters (– – – –) as regards the threshold value (S_r) and duration of effect (T_{oe}) in the oestrus test.

II. *Uterus growth test*

3-Mono-esters. As in the oestrus test the lower aliphatic 3-mono-esters show also in the uterus growth test (Fig. 4) first of all only a slight increase of effect

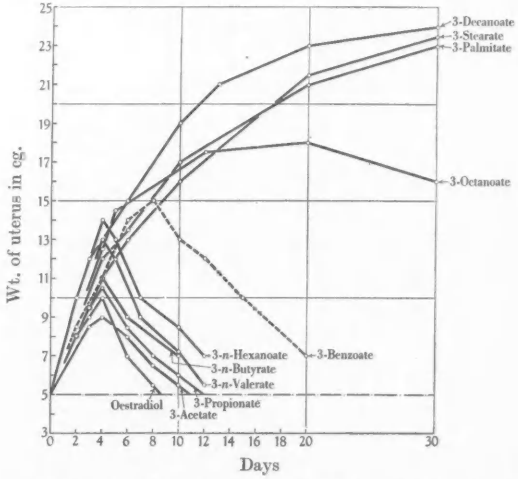

Fig. 4. The effects of aliphatic 3-mono-esters of oestradiol in comparison with oestradiol and its 3-benzoate on the weight of the uterus of young rats after subcutaneous injection of a total dose of 50 γ of hormone given in equal parts on two consecutive days.

with the increasing length of the acid radical. On the other hand, the 3-octanoate, and particularly the higher esters, exhibit more pronounced effects. As regards effect the 3-benzoate is between the 3-hexanoate and 3-octanoate. The lower esters up to the 3-hexanoate attain their maximum effect on the 4th day. With the 3-octanoate it is delayed to the 20th day, the maximum effect of the higher esters appearing still later.

17-Mono-esters. The intensive action of the esters substituted in the 17-position is very obvious (Fig. 5). Already with the 17-propionate the intensity of effect obtained with the 3-benzoate is reached, yet as regards duration the former is superior (more than 30 days against 20 days). From the 17-*n*-valerate onwards all higher esters show particularly pronounced effects; even after 30 days the action is still maintained. In comparison with the 3-mono-esters the maximum effect of the corresponding lower 17-mono-esters is delayed. Generally it may be said that the higher the activity of the ester the later the maximum is reached.

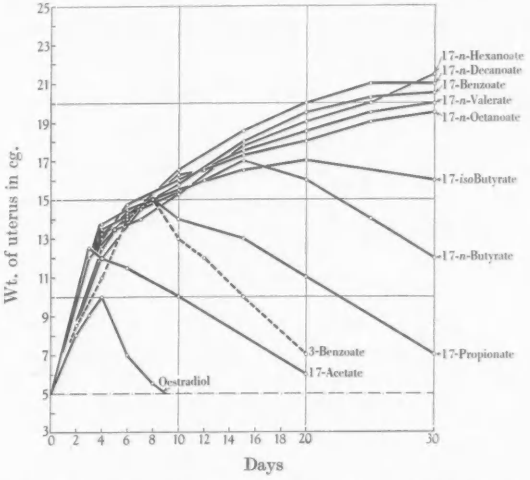

Fig. 5. The effects of 17-mono-esters of oestradiol in comparison with oestradiol and its 3-benzoate on the weight of the uterus of young rats after subcutaneous injection of a total dose of 50γ of hormone given in equal parts on two consecutive days.

The already mentioned carbonates were examined also as regards their effects in the uterus growth test. The 17-monoethylcarbonate with an output of activity of 204 cg.-days was twice as active as the 17-monomethylcarbonate (approx. 100 cg.-days). The diethylcarbonate of oestradiol shows an action similar to that of the oestradiol-17-*iso*butyrate.

III. *Comparison of the effects on the uteri of castrated and uncastrated animals*

Up to the present our results have been mainly obtained with young uncastrated rats. Experiments with oestradiol-17-octanoate and -dipropionate on a great number of castrated rats demonstrate that during the comparable interval of the first 30 days the corresponding values obtained agree in an astonishing way (see Fig. 6), especially considering the different weights of the

two kinds of animals. However, it should be taken into account that the average weight of the uteri of the castrated untreated rats which we used was approximately the same as that of the young rats. In any case the use of young uncastrated rats for the testing work during the first 30 days is quite justified.

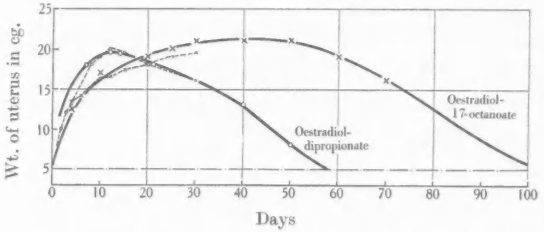

Fig. 6. Comparison of the effects of oestradiol-dipropionate and oestradiol-17-octanoate on the weight of the uteri of young uncastrated rats (– – –) and of castrated rats (————) after subcutaneous injection of a total dose of 50γ hormone given in equal parts on two consecutive days.

IV. *Characterization of the effect of the investigated compounds*

As in the previous publications the effects of the compounds dealt with in this paper have been characterized systematically, in so far as complete data are available, according to their output of activity, A, onset of the maximal effect, T, etc. With all higher esters the output of activity is above 300 cg.-days and attains quite considerable values.

Table II

	Absolute values					Relative values: Based on $G_o=5$ cg.		Relative values: Based on oestradiol =1
Substance	A cg.-days	D_{max} cg.	t_{max}	T days	D_m cg.	d_{max} %	d_m %	c
Oestradiol	20·3	5·0	4	9	2·3	100	46	1·0
-3-acetate	22·6	4·0	4	11	2·0	80	40	1·1
-3-propionate	30·0	5·5	4	12	2·5	110	50	1·5
-3-*n*-butyrate	37·2	6·0	4	13*	2·9	120	58	1·8
-3-*n*-valerate	46·0	8·0	4	13	3·5	160	70	2·3
-3-*n*-hexanoate	59·6	9·0	4	14*	4·2	180	84	2·9
-3-benzoate	118·8	10·0	8	24*	4·9	200	99	5·9
-17-acetate	86·0	7·5	3	22	3·9	150	78	4·2
-17-propionate	194·0	10·0	8	34*	5·7	200	114	9·6
-17-*n*-butyrate	327·0	12·0	15	46*	7·1	240	142	16·1
-17-*n*-octanoate	1100·0	16·0	30	100*	11·0	320	220	54·0
-17-methylcarbonate	100·0	8·0	7	24*	4·2	160	84	4·9
-17-ethylcarbonate	204·0	9·0	7	37*	5·5	180	110	10·1

* Estimated.

V. *Histological observations after administration of oestrone and oestradiol esters*

It is known that the injection of follicular hormone into female rodents causes characteristic modifications in the structure of the mucous membrane of the uterus and vagina.

The modification produced by esters of oestrone and oestradiol depends on the dose given and on the intensity and duration of effect of the derivatives concerned. After giving rats 50γ (administered subcutaneously in two equal doses on

consecutive days) of one of the esters investigated by us, the histological picture of the uteri shows a pronounced hypertrophy of all tissue layers so long as the typical cornified non-nucleated epithelial cells are present in the vaginal smear. Macroscopic examination shows that this organ is enlarged considerably and during the first days of the experiment is filled with liquid and mucus. With the shorter-acting esters the liquid diminishes from about the 6th to the 15th day and from the 15th to the 20th day in the case of the longer-acting esters.

When the effect is mild, the epithelium of the mucous membrane consists first of all of high cellular elements in a single layer. With esters exhibiting a more intense and more prolonged effect other modifications occur which are to be seen particularly in the epithelium. Whereas in the musculature and the mucous membrane a large number of eosinophil leucocytes appear, characteristic changes are perceptible on the epithelium. In the place where single-layered cylindrical epithelium was to be expected, with esters having a prolonged effect several strata of partly keratinized cells of very high cylindrical epithelium ("vertical keratinization" according to Freud [1937]) are found. The multiplicity of layers can be very pronounced at certain places yet the cylindrical epithelial character always remains. An infiltrative growth with destruction of the limiting membrane or deep proliferation of solid epithelial cones, as described by Selye *et al.* [1935] after administration of high doses of follicular hormone was not observed in our experiments. When the effect of the hormone ceases then the cornified non-nucleated cells in the vaginal smear disappear, the uterus atrophies and instead of the cornified epithelial cells nucleated epithelial cells appear; these are finally replaced by normal cylindrical epithelial cells.

Only in isolated cases and with esters of oestradiol having a very prolonged effect, were we able to observe in castrated rats a change of the single-layered cylindrical epithelium of the mucosa into a multiple-layered pavement epithelium as described by Selye *et al.* [1935], Pierson [1935], Grumbrecht [1935], Korenchevsky [1937], Szarka & Rechnitz [1938], Dessau [1938] etc.

We must mention that in our rats, even when using high doses of esters of oestradiol having very prolonged action, malignant tumours were never observed.

Note. After completing this work we became aware of a publication by A. Fischer [1938] entitled: "Ueber die biologische Wirkung der Follikelhormon-Derivate." Probably following on our own publications [Miescher, 1937; Miescher *et al.* 1937, 1, 2, 3; 1938, 1, 2], this author also investigated esters of oestrone and oestradiol, particularly oestradiol-17-mono-esters and determined, as we did, besides the threshold value in the oestrus test also the duration of the oestrus and the effect on the uterus. The oestrus threshold values ascertained on rats agree with those communicated by us. To determine the duration of oestrus, Fischer gave a single dose of 1γ to castrated mice and to ascertain the effect on the uterus he gave within 4 days 4 injections of 1·7γ each to young rats and weighed the dried uterus on the 5th day. Fischer did not obtain satisfactory results with the uterus test and therefore he recommends the seminal vesicle test.

As is shown from the comparison with our results the experiments made by Fischer are to be considered as inadequate for the judgment of the effect of sex hormone compounds. The efficacy of a substance cannot be judged on the results of tests with small doses alone. A proper conclusion regarding the total activity of such a compound can only be made when higher doses are also taken into consideration. In the uterus growth test (and moreover also in the seminal vesicle test) the time course of the swelling and the subsidence of the swelling of the organ after injection must be considered. When this test is carefully carried out there is, contrary to the opinion of Fischer, a certain parallelism between the duration of the effect in the oestrus test and in the uterus growth test.

SUMMARY

1. Nine aliphatic 3-mono-esters, ten aliphatic 17-mono-esters and the 17-monobenzoate of α-oestradiol were tested by subcutaneous injection into the rat by the oestrus test and uterus growth test and the results compared with those obtained with oestradiol-3-benzoate.

2. *Oestrus test.* In both series of esters containing a fatty acid radical the threshold values are, up to the octanoates, comparatively low and are similar to that of free α-oestradiol (0·4 γ). From the octanoates they increase rapidly but do not exceed 7 γ for the esters investigated. The threshold values of the 17-mono-esters are generally somewhat higher than those of the corresponding 3-mono-esters. A prolonged effect is obtained particularly with the higher esters. In this respect all the 17-esters investigated are much superior to the corresponding 3-esters; the 17-octanoate exhibiting the most intense and prolonged effect even with small doses.

3. *Uterus growth test.* In this test also 17-mono-esters, particularly the lower esters, prove to be superior to the corresponding 3-mono-esters. The results of experiments with oestradiol dipropionate and the 17-mono-octanoate on uncastrated young and on castrated adult rats agreed in an astonishing way.

4. *Histological observations.* The single-layered cylindrical epithelium changes under the prolonged influence of hormone into several strata of partly keratinized cells of very high cylindrical epithelium ("vertical keratinization"). An infiltrative growth with destruction of the limiting membrane or deep proliferation of solid epithelial cones did not occur with our method of testing. Malignant tumours were not observed. When the effect of the hormone ceased a return to normal followed.

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CLXX. THE ACID-SOLUBLE PIGMENT OF RED HUMAN HAIR

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The pigment responsible for the distinctive colour of human red hair appears to be unknown. Text-books contain conflicting statements in regard to the question. Morse [1927] states that "red hair is a diluted black". If this is true, it becomes difficult to explain the many shades of brown which various samples of hair exhibit. On the other hand, Bodansky [1934] states: "In addition to melanin, the presence of lipochrome has been described in skin and hair, to which is attributed the characteristic red coloration which is often seen in hair." The only lipochrome discovered by Tutschku [1923] in horse hair, however, had a yellow-green colour and was present in hair of all shades except white. Neither Morse nor Bodansky quotes literature references in support of the statement contained in his text.

Zwicky & Almasy [1935] have reported that the alkali-soluble pigments of red hair, black hair and melanomas of horses cannot be distinguished spectroscopically. The present author does not agree with this conclusion, since inspection of their data shows that the ratios of the extinction coefficients of the black hair extracts and the red hair extracts are not constant. That is, the two spectral curves cannot be made to coincide at any concentrations of the pigments.

Neumann [1937] has recently made a study of the pigments of rabbit hair. Three pigments, described as yellow, brown and black, were found. The yellow pigment has the lowest molecular weight and the lowest nitrogen content while the black pigment has the highest molecular weight and nitrogen content.

Experimental

Extraction of acid-soluble pigment. Samples of red human hair were thoroughly washed with successive portions of 0·1 *N* NaOH, 0·1 *N* HCl and distilled water. The hair was then partly dried between filter papers, after which it was extracted with boiling 0·1 *N* HCl for periods varying from 3 to 10 days. During the first 2 days the refluxing solution remained colourless; from the third day on, however, coloration was present. The extract was concentrated by boiling and filtered to remove the hair residue and some protein which precipitated. The filtrates were clear; dilute solutions were coloured reddish yellow and more concentrated solutions were red-brown.

In order to demonstrate that the pigment extracted by this procedure was not produced by the chemical treatment, samples of black hair and of synthetic dopa-melanin (prepared by the oxidation of dopa [Arnow, 1938]) were refluxed separately with 0·1 *N* HCl for varying lengths of time. The red pigment was never produced in these experiments. Apparently black hair does not contain an acid-soluble pigment, and such a pigment cannot be produced from dopa-melanin by prolonged boiling with dilute HCl.

Preparation of synthetic pigment. Solutions of dopa-melanin in *N* NaOH were placed in 75 ml. test tubes. Air was bubbled through these solutions for several days. Excess HCl was then added and the precipitated dopa-melanin was removed by centrifuging and filtration. The resulting solution resembled in colour the extract of red hair.

The pigment produced in this manner from dopa-melanin appears to be an oxidation product. Solutions of dopa-melanin in *N* NaOH which are kept in containers evacuated with a water pump are stable more or less indefinitely. Similar solutions exposed to air slowly produce the acid-soluble pigment.

The experiments of Dulière & Raper [1930] indicate that melanin can be oxidized with oxygen in alkaline solution. On theoretical grounds, the production of melanin from tyrosine by tyrosinase should require 5 O per mol. of tyrosine. At *p*H 6 this value was found, by the above authors, but 5·23 O were used at *p*H 8. Moreover, "if a few drops of 30 % KOH were added to the solution in the experimental flask after all enzyme action had ceased, a further oxygen uptake was observed. This occurred outside the limits of *p*H at which tyrosinase is active and was probably due to production of oxidation products of melanin itself by atmospheric oxygen."

Comparison of natural and synthetic pigments. (1) Both pigments are soluble in acid, neutral and alkaline solutions. The term "acid-soluble pigment" has been used in this paper to distinguish the coloured compounds from melanin (and perhaps other hair pigments), which is insoluble in dilute mineral acid solutions.

(2) Both pigments exhibit more colour in alkaline solution than in equal concentration in acid solution. The degree of acidity or alkalinity can vary within wide ranges without affecting the colour, provided that the concentration of pigment is unaltered.

(3) The visible absorption spectra of the two pigments in either acid or alkaline solution agree qualitatively within experimental error (see Fig. 1). The absolute concentrations are not known, since neither substance has yet been isolated in pure form. It is possible, however, to adjust the solutions to equal concentrations as determined either with the spectrophotometer or with a Duboscq colorimeter. A Bausch and Lomb spectrophotometer was used in making the measurements recorded in Fig. 1. In accordance with Lambert's law, the extinction coefficient is defined by the expression,

$$\text{extinction coefficient} = \frac{1}{d} \log_{10} \frac{I_0}{I},$$

where I is the intensity of light after passage through d cm. of solution, and I_0 is the intensity of the light entering the solution. According to Beer's law, for any given wave-length this extinction coefficient is proportional to concentration, if d is maintained constant. The absorption vessels used in making these measurements were 2·5 cm. long.

Fig. 1 shows the increase in colour in alkaline solution, since the concentration of pigment is the same in both solutions. The colour in acid solution is not qualitatively identical with that in alkaline solution. This is illustrated in Fig. 1 by a plot of the ratio of the extinction coefficients in alkaline solution to those in acid solution as a function of the wave-length. This would result in a straight line parallel with the abscissa if the colours were qualitatively identical.

(4) When acid solutions of the two pigments are shaken with *n*-butyl alcohol, a portion of the pigment enters the alcohol layer. If the experiment is

repeated, substituting alkaline solutions for acid solutions, no pigment enters the alcohol layer.

(5) Solutions of both pigments pass through dialysing membranes (Visking casing).

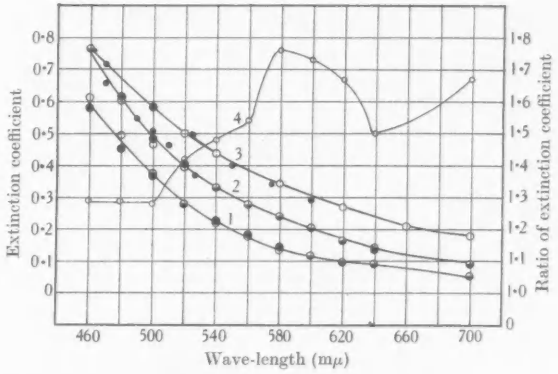

Fig. 1. Absorption spectra data.

Curve 1, pigments in hydrochloric acid solution.
- o solution of pigment extracted from red human hair.
- ● solution of synthetic pigment.
- • points calculated from data of Zwicky & Almasy [1935].

Curve 2, pigments in sodium hydroxide solution.
- o, ●, • } As for curve 1.

Curve 3, dopa-melanin and black horse hair pigment in alkaline solution.
- o dopa-melanin in 0·02*N* NaOH (1·37 mg. per l.).
- • points calculated from data of Zwicky & Almasy [1935].

Curve 4, ratio of extinction coefficients of the pigment of red human hair (and of synthetic pigment) in alkaline solution and in acid solution.

Discussion

The evidence presented above suggests that the characteristic pigment of red human hair is an oxidation product of melanin. The strongest evidence of the identity of the synthetic and natural pigments is afforded by the spectral data. It is true that the evidence presented indicates only that the absorption spectra of the two pigments agree qualitatively with each other. It seems unlikely, however, that two chemically different substances could have the same qualitative spectra in both acid and alkaline solutions, particularly in view of the fact that the spectra are both quantitatively and qualitatively different in these solvents. In respect to the other physical properties recorded in this paper, the pigments also agree with each other.

The data of Zwicky & Almasy [1935], mentioned earlier, give some support to the present author's data. If the concentrations of the solutions studied by them are adjusted to those used in this paper by multiplying the extinction coefficients calculated from their data by a suitable constant, it is found that the spectral curve for the pigment of red horse hair agrees qualitatively with that for red human hair. This is indicated in Fig. 1, in which is also indicated the interesting fact that the spectral curve for the pigment of black horse hair

agrees qualitatively with that for dopa-melanin. An inspection of these graphs will show that it is impossible to make the curve for dopa-melanin (or black horse hair pigment) coincide with that for an alkaline solution of the pigment of red hair (or oxidized dopa-melanin) by multiplication of the values of the ordinates by a constant. For this reason, the author cannot agree with the conclusion expressed by Zwicky & Almasy, that "Das schwarze Pigment der einen Art, das Pigment roter Haare...erwiesen sich im gelösten Zustand als spektroskopisch ununterscheidbar". The expression "der einen Art" is used because a pigment whose absorption spectrum was obviously different from that of melanin was found in certain samples of horse hair.

Some protein is extracted along with the acid-soluble pigment, as indicated by the fact that the hair extract gives the usual protein colour tests, whereas solutions of the pigment prepared from dopa-melanin do not.

The fact that the colour of the pigments deepens in alkaline solution suggests that the compounds are weakly acidic in character. The increased colour would then be explained by the increased ionization in alkaline solution. This view is strengthened by the finding that butyl alcohol will extract them from acid aqueous solution, but not from alkaline aqueous solution.

Summary

If red human hair is extracted with boiling 0·1 *N* hydrochloric acid, a red-brown pigment is obtained in solution. This pigment has the physical properties of a pigment prepared by the mild oxidation of dopa-melanin in alkaline solution. It is suggested that the distinctive colour of red hair is due to the presence in such hair of an oxidation product of melanin.

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CLXXI. THE EFFECT OF BODY STORES ON THE EFFICIENCY OF CALCIUM UTILIZATION

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Many Ca and P balance experiments with animals and man have been carried out during the last 35 years. The resulting data are rather difficult to interpret because of much variation from unknown causes. It occurred to the writer that possibly the degree of Ca "saturation" of the tissues was a factor influencing the efficiency with which an individual utilized his dietary Ca.

It has been shown by Fairbanks & Mitchell [1936] that a low Ca content of the body brought about by subsistence on a diet poor in this element predisposes to a subsequent high retention of Ca when liberal amounts are furnished in the diet. In their experiment, the Ca content of the diet was 1·25% in the experimental period which followed a preliminary period on a low Ca diet. The 1·25% Ca level is so much in excess of the Ca requirement for maximum storage, however, that the animals were able to replenish their depleted Ca stores even with a low percentage retention. On the basis of the intake and balance figures given in their paper, the writer has calculated the average percentage retention for the animals that had been on the low diet in a previous period: this was found to be only 24%. Hence, this experiment does not elucidate the question as to whether an individual becomes more efficient in the utilization of the available dietary Ca in times of stress.

In order to test this point it is essential that the Ca furnished in the diet in a test period, following a preparatory period on a low Ca diet, be below the requirement for maximum storage. It was felt that the point was of sufficient importance in connexion with the determination of the Ca requirements of man and animals to justify experimental enquiry.

First experiment

This experiment was conducted according to the paired feeding method. The food intake of a pair was restricted to the amount consumed by the member eating the less.

Six young hooded male rats were selected and paired, the two members of a pair being of the same litter and as nearly alike as possible. Four of the animals were 33 and two were 30 days old when they were started on the balance experiment.

A basal diet of the following composition was fed:

Yeast	...	...	5
Casein	...	...	5
Wheat gluten	...		15
Corn starch	...		60
Butter fat	...	...	12
Salt mixture	...		3

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The salt mixture was De Loureiro's [1931] with calcium phosphate omitted as adapted by Henry & Kon [1937], and was made up from B.D.H. "Analar" reagents.

This basal diet contained 0·10 % Ca and 0·19 % P; its P content was adjusted to 0·4 % by addition of K_2HPO_4. In the preliminary period the Ca was fed at two levels, namely 0·15 and 0·8 %, one member of a pair being fed the 0·15 % Ca diet, the other the 0·8 % Ca diet. The Ca content was regulated by addition of $CaCO_3$. In all cases the odd number rats received the low Ca diet in the preliminary period.

The diets were fed in four 7-day balance periods. The animals were kept in Hopkins metabolism cages made of stainless steel. At the end of each 7-day collection period the cages, beakers and funnels were rinsed in 5 % HNO_3 and the washings added to the urine. The urine was evaporated to dryness and ashed before being analysed.

The 4 weeks' preliminary period was followed by a 5 weeks' test period during which the Ca content of the diet was adjusted to the 0·4 % level for both members of a pair. At the end of the test period the animals were killed, the intestinal tract was removed and the carcass analysed for Ca and P.

Results

The food intake and gain in live weight are given in Table I. The average daily gain in live weight in the preliminary period was 1·5 g. for the animals on the low Ca diet and 1·7 g. for those on the high Ca diet; in the test period the live weight gain was almost the same in both groups, averaging 2·2 g. daily. The relatively low gain in the preliminary period was due to lack of appetite in the animals on the low Ca diet. There was not a single instance of refusal of food in the animals on the high Ca diet. On the low Ca diet, on the other hand, refusals occurred frequently, although the attempt was made not to feed more than the amount which would be eaten within 24 hr.

The food consumption improved in the test period and naturally a better growth was obtained. The figures for growth indicate a better food utilization on the high Ca diet, which was to be expected in the light of the studies by Toepfer & Sherman [1936].

Table I. *Food consumption and live weight gain (g.) in first exp.*

	Pair I		Pair II		Pair III	
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Initial live wt.	39	36	48	44	40	41
Final live wt.	153	150	167	169	165	175
Carcass wt.	133	128	145	147	143	153
Av. daily gain in preliminary period	1·3	1·5	1·4	1·6	1·8	2·0
Av. daily gain in test period	2·2	2·1	2·3	2·3	2·2	2·2
Daily food consumption in preliminary period	5·1	5·1	4·8	4·8	6·0	6·0
Daily food consumption in test period	8·7	8·7	9·2	9·2	9·3	9·3

The average Ca and P balances for the four weekly preliminary periods are given in Table II. On an average the animals on the low Ca intake stored 191 mg. Ca and 179 mg. P during the 4 weeks as compared with an average storage of 570 mg. Ca and 293 mg. P by their pair mates on the high Ca diet.

Table II. *Mean storage of Ca and P in first exp.*

		Ca					P				
					Stored					Stored	
	Rat no.	Intake mg.	Faeces mg.	Urine mg.	mg.	%	Intake mg.	Faeces mg.	Urine mg.	mg.	%
Weekly av. in preliminary period	1	53·2	2·5	1·7	49·0	92·1	142·0	12·9	86·9	42·2	29·7
	2	284·0	128·7	20·6	134·7	47·4	142·0	59·1	13·2	69·7	49·1
	3	49·9	12·6	1·4	35·9	71·9	133·0	14·2	85·6	33·2	25·0
	4	266·0	119·0	22·0	125·0	47·0	133·0	53·2	17·8	62·0	46·6
	5	63·0	3·2	1·4	58·4	92·7	168·0	13·2	96·0	58·8	35·0
	6	336·0	148·0	20·3	167·7	49·9	168·0	61·2	19·1	87·7	52·2
Weekly av. in test period	1	244·8	50·4	7·8	186·6	76·2	244·8	38·1	87·5	119·2	48·7
	2	244·8	116·6	8·4	119·8	48·9	244·8	71·5	88·3	85·0	34·7
	3	256·8	63·7	8·6	184·5	71·8	256·8	46·5	89·7	120·6	47·0
	4	256·8	137·2	21·3	98·3	38·3	256·8	74·6	83·9	98·3	38·3
	5	259·2	68·5	7·5	183·2	70·7	259·2	45·7	100·0	113·5	43·8
	6	259·2	109·2	8·1	141·9	54·7	259·2	65·5	94·4	99·3	38·3

At the start of the experiment two male rats similar to the experimental animals were analysed for Ca and P and found to contain 0·52 % Ca and 0·40 % P. In Table III will be found calculations of the Ca and P contents of the experimental animals at the end of the preliminary period, based on the assumption that these animals had the same initial Ca and P percentages as those of the two animals analysed. The average Ca and P contents of the rats on the low Ca diet worked out at 411 and 348 mg. respectively. The corresponding figures for the animals on the high Ca diet were 779 mg. Ca and 454 mg. P. The rats on the high Ca diet thus contained 90 % more Ca and 30 % more P than their pair mates on the low Ca diet, but in spite of the low Ca content of these latter animals, they seemed to be in excellent condition. The only difference between the pair mates which could be detected by the eye was a difference in the incisor teeth. The incisors of the depleted animals were opaque with white chalky patches. The incisors of the replenished animals were transparent with a uniform brownish lustre.

The balance data for the test period are given in Table II. The table shows that the depleted animals made up for previous shortage by a more efficient utilization of the Ca and P in the food. The depleted animals stored on an average

Table III. *Ca and P stored and Ca and P content of the carcasses at the end of first exp.*

Rat no. ...	1		2		3		4		5		6	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
Initial content, mg.	203	156	187	144	250	192	229	176	208	160	213	164
Stored preliminary period, 28 days, mg.	196	169	539	279	143	133	500	248	234	235	671	351
Stored test period, 35 days, mg.	933	596	599	425	923	603	492	491	916	567	710	496
Total stored, mg.	1332	921	1325	848	1316	928	1221	915	1358	962	1594	1011
Found in carcass, mg.	1334	899	1292	842	1365	906	1370	906	1370	906	1538	1000
Difference, mg.	−2	+22	+33	+6	−49	+22	−149	+9	−12	+56	+56	+11
Difference %	0·1	2·4	2·5	0·7	3·6	2·3	10·9	1·0	0·9	6·3	3·6	1·1
Ca and P as % of net wt.	1·00	0·68	1·01	0·66	0·94	0·58	0·93	0·62	0·96	0·63	1·01	0·65
Ca/P ratio of minerals stored in preliminary period	1·2		1·9		1·1		2·0		1·0		1·9	
Ca/P ratio of minerals stored in test period	1·6		1·4		1·5		1·0		1·6		1·4	
Ca/P ratio of minerals in carcass	1·5		1·6		1·5		1·5		1·5		1·5	

924 mg. Ca and 589 mg. P during the test period as compared with 600 mg. Ca and 471 mg. P stored by their pair mates. During the entire experiment the storage was nearly equal in the pair mates. The average figures for the depleted animals are 1115 mg. Ca and 768 mg. P as compared with 1170 mg. Ca and 763 mg. P in the replenished ones.

The Ca and P contents of the animals as calculated from the balance figures showed good agreement with the Ca and P found by analyses of the carcasses, as will be seen in Table III. The Ca data for number 4 furnished an exception to this rule and showed a deviation of 10·9 %. The deviation for the other animals ranged from 0·1 to 6·3 %. In the preliminary period the animals on the high Ca intake lost a large percentage of the Ca in the faeces. The average for the three animals was approximately 45 %. The animals on the low Ca intake excreted only very small amounts of Ca in the faeces; two of the animals excreted about 5 %, the third about 25 % of the food Ca in the faeces. In the amount of Ca lost in the urine there was also a marked difference between the pair mates. In the preliminary period the loss was from 6·1 to 8·3 % of the intake for the animals on the high Ca intake. On the low Ca intake the corresponding figures were from 2·3 to 3·2 %. Two of the animals on the low intake stored approximately 92 % of the food Ca, the third stored 72 %. On the high Ca diet the storage was from 47 to 50 %.

The P excretion also showed a striking difference between the pair mates. On the low Ca diet the P was excreted mainly in the urine, on the high Ca diet mainly in the faeces. In the urine the averages were from 57 to 64 % on the low Ca diet and only from 9 to 13 % on the high Ca diet. In the faeces the excretion of P ranged from 36 to 42 % on the high Ca diet and only from 8 to 11 % on the diet low in Ca. The difference in P storage was about 20 % in favour of the animals on the high Ca intake.

In the following 5 weeks' test period when the pair mates were fed the same diet in equal amounts, the depleted animals utilized the dietary Ca more efficiently than did their replenished pair mates. This was mainly brought about by better absorption from the intestine. The difference in the urinary Ca was comparatively slight, but this difference was in favour of the depleted animals too. The replenished animals lost from 42 to 53 % of the Ca in their faeces as compared with 21 to 26 % in the case of the depleted ones.

The P excretion in the urine was nearly equal for the pair mates. In the faeces on the other hand there was a difference of about 10 % in favour of the depleted animals.

Second experiment

This experiment was conducted in much the same way as the first one, the only differences being shorter balance periods and a lower Ca content of the

Table IV. *Food consumption and live weight gain (g.) in second exp.*

	Pair I		Pair II		Pair III	
	No. 7	No. 8	No. 9	No. 10	No. 11	No. 12
Initial live wt.	50	33	52	53	51	43
Final live wt.	146	153	150	159	159	161
Carcass wt.	135	142	138	147	147	148
Av. daily gain in preliminary period	2·0	2·7	1·8	2·0	2·0	2·5
Av. daily gain in test period	2·5	3·0	2·9	3·0	3·1	3·1
Av. daily food consumption in preliminary period	7·0	7·0	6·9	6·9	7·6	7·6
Av. daily food consumption in test period	10·4	10·4	10·2	10·2	11·0	11·0

ration during the test period. The preliminary period and the test period each lasted for only 3 weeks and the Ca level in the test period was adjusted to 0·25%. A carmine marker was administered at the end of the preliminary period in order to effect a better separation between the faeces from the two periods.

The six male rats selected for this experiment were 33 days of age at the start. The food intake and gain in live weight as shown in Table IV were higher than in the previous experiment. The animals in this experiment gained only slightly less in 6 weeks than the animals in the first experiment did in 9 weeks.

Results

The balance data are given in Table V. The average storage for the preliminary period was 212 mg. Ca and 154 mg. P on the low Ca diet. On the high Ca diet the average storage was 545 mg. Ca and 280 mg. P. Assuming that these rats had the same initial percentages of Ca and P as the two rats analysed in the first experiment, the difference in Ca and P contents of the rats on the high and the low Ca diets at the end of the preliminary period was found to be less than in the first experiment. The calculated figures show that the animals on the high Ca diet contained 60% more Ca and 25% more P than did their pair mates on the low Ca diet. An examination of the incisor teeth did not reveal such a striking difference as that found in the first experiment, although the incisors of the depleted animals were definitely poorer than those of the replenished ones.

Especially in the first week of the test period the depleted animals proved to be more efficient in the utilization of dietary Ca. The total Ca excretion in the faeces and the urine was more than three times as high in the replenished animals as in their depleted pair mates. The difference in the Ca excretion between the pair mates in the preceding period, however, was so large that if only a small amount of faeces from the diet of the preliminary period had been collected at the beginning of the test period, the Ca contained herein would easily account for the higher Ca content of the faeces from the replenished rats in the first week of the test period. The use of a marker does not entirely dispose of this possibility since the marker does not effect a perfect separation. The P excretion, however, strongly indicated that no excreta from the previous period had been collected in the test period. In the week prior to the change in diet the P excreted in the faeces was 5–6 times larger in rats on the high than in those on the low Ca diet. In the urine the reverse condition held true. On the low Ca diet 3–4 times more

Table V. *Mean storage of Ca and P in second exp.*

		Ca					P				
					Stored					Stored	
	Rat no.	Intake mg.	Faeces mg.	Urine mg.	mg.	%	Intake mg.	Faeces mg.	Urine mg.	mg.	%
Weekly av. in preliminary period	7	74·0	3·7	1·3	69·0	93·2	197·3	16·9	131·1	49·3	25·0
	8	394·6	178·7	42·9	173·0	43·8	197·3	80·4	19·9	97·0	49·2
	9	72·5	3·1	1·6	67·8	93·5	193·3	16·2	127·9	49·2	25·5
	10	386·6	190·4	27·2	169·0	43·7	193·3	78·8	35·3	79·2	41·0
	11	79·5	2·7	1·4	75·4	94·8	212·0	17·8	138·7	55·5	26·2
	12	424·0	185·0	36·0	203·0	47·9	212·0	81·2	27·6	103·2	48·7
Weekly av. in test period	7	182·5	14·0	2·6	165·9	90·9	292·0	26·7	140·0	125·3	42·9
	8	182·5	22·5	6·6	153·4	84·1	292·0	26·9	134·8	130·3	44·6
	9	179·2	10·8	2·6	165·8	92·5	286·6	24·7	126·9	135·0	47·1
	10	179·2	25·2	9·2	144·8	80·8	286·6	30·3	136·2	120·1	41·9
	11	191·6	10·7	3·4	177·5	92·6	306·6	25·7	145·7	135·2	44·1
	12	191·6	20·0	7·1	164·5	85·9	306·6	28·6	143·1	134·9	44·0

P was excreted in the urine than on the high Ca diet. During the first week of the test period the pair mates excreted nearly equal amounts of Ca and P in the faeces and urine.

In the following 2 weeks the depleted animals stored only slightly more Ca than did their pair mates. The difference was mainly brought about by a smaller Ca loss in the faeces though the urinary Ca excretion also favoured the depleted animals. In P metabolism there was no significant difference between the pair mates.

The agreement between the Ca and P contents of the rats calculated on the basis of balance data and the Ca and P contents found by analyses of the carcasses was very close (Table VI). The maximum deviation was only 2·5 % for Ca and 4·0 % for P.

Table VI. *Ca and P stored and Ca and P contents of the carcasses at the end of the second exp.*

Rat no. ...	7		8		9		10		11		12	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
Initial content, mg.	260	200	172	132	270	208	276	212	265	204	224	172
Stored preliminary period, 21 days, mg.	207	148	519	291	203	148	507	238	226	167	609	310
Stored test period, 21 days, mg.	498	376	460	391	497	405	434	360	532	406	493	405
Total stored, mg.	965	724	1151	814	970	761	1217	810	1023	777	1326	887
Found in carcass, mg.	990	712	1166	807	963	732	1198	801	1011	759	1300	891
Difference, mg.	−25	+12	−15	+7	+7	+29	+19	+9	+12	+18	+26	−4
Difference %	2·5	1·7	1·3	0·9	0·7	4·0	1·6	1·1	1·2	2·4	2·0	0·4
Ca and P as % of net wt.	0·73	0·53	0·82	0·57	0·70	0·53	0·82	0·54	0·69	0·52	0·88	0·60
Ca/P ratio of minerals stored in preliminary period	1·4		1·8		1·4		2·1		1·4		2·0	
Ca/P ratio of minerals stored in test period	1·3		1·2		1·2		1·2		1·3		1·2	
Ca/P ratio of minerals in carcass	1·4		1·4		1·3		1·5		1·3		1·5	

During the preliminary period on the low Ca diet the animals stored on an average 212 mg. Ca and 154 mg. P. The corresponding figures for their pair mates were 545 mg. Ca and 280 mg. P. In the test period the average storage was 509 mg. Ca and 396 mg. P for the depleted animals, as compared with 462 mg. Ca and 385 mg. P for their replenished pair mates.

In the test period the depleted animals stored 92 % of the Ca and 44·7 % of the P. The corresponding figures for their pair mates were 83·6 and 43·5. The replenished animals excreted about 6 % more Ca in the faeces and about 2·5 % more Ca in the urine than did their pair mates. It must be kept in mind, however, that the difference between the pair mates decreased after the first week of the test period.

Discussion

Data reported in the literature indicate that 0·4 % Ca in the diet is inadequate for maximum storage when growth is normal. The rate of growth obtained in these experiments, however, was below normal and the possibility that the replenished animals were able to keep their tissues "saturated" with Ca must be considered.

The analyses of the carcasses support the view that the animals in the first experiment did keep their tissues "saturated" with Ca on the 0·4 % Ca level. The balance data on the other hand show a much smaller Ca content of the live

weight increase in the test period than in the preliminary period on the 0·8 % Ca level. Calculated on the basis of the balance data and gain in live weight the live weight increase contained 1·19 % Ca and 0·61 % P in the preliminary period. In the test period on 0·4 % Ca the live weight increase contained 0·78 % Ca and 0·61 % P. The high Ca figure for the preliminary period is of course partly brought about by the low initial Ca content of the animals, but even when allowance is made for the increase in Ca percentage due to increase in age, there is still a difference left between the two periods which may be explained as resulting from the inadequacy of the Ca level of the diet in the test period.

In the second experiment the Ca level was so far below the requirement that the replenished animals were rapidly depleted of Ca, which undoubtedly contributed to improved utilization as the test period advanced. The carcasses of the depleted animals contained 0·71 % Ca as compared with 0·84 % in the replenished animals. The corresponding figures for the first experiment were 0·97 and 0·98.

From the findings of the two experiments it seems justifiable to draw the conclusion that the degree of Ca "saturation" has an effect on the efficiency of Ca utilization. The efficiency is greater with depleted than with replenished stores. This must be taken into consideration when interpreting mineral balances in animals and man. Equilibrium or positive balance is not necessarily proof of adequacy of intake. It might be in part a reflexion of the level of previous mineral nutrition. In other words, an intake which will barely maintain equilibrium with depleted stores might not suffice to maintain equilibrium when the stores are filled. In this connexion one might naturally ask if it is desirable to keep the Ca stores filled. Our present knowledge is not sufficient to answer this question, but it does seem likely that Ca stores may be considerably reduced and Ca utilization consequently improved without any harmful effect to the individual.

Under the conditions of this experiment, the P excretion in the faeces was determined to a considerable extent by the amount of Ca present. In the preliminary period of both experiments, on the high Ca diet about 40 % of the ingested P was lost in the faeces and 8–10 % in the urine. The corresponding figures for the low Ca diet are 8–10 % in the faeces and 60–70 % in the urine. McGowan [1933] showed with rabbits that a shift in the P excretion from the urine to the faeces occurred after addition of $CaCO_3$ to the diet but that the retention was better with $CaCO_3$ than with dicalcium hydrogen phosphate or steamed bone flour. He claimed that the neutralization of gastric HCl by $CaCO_3$ or other basic salts was the cause of the lowered Ca and P absorption from the intestine. In the test periods of the present experiment it was not the neutralization of gastric HCl which caused an increased loss of P in the faeces. The pair mates were fed the same diet but none the less the replenished animals lost more Ca and P in the faeces than did their depleted pair mates. This fact seems best explained on the assumption that the large amount of Ca present in the faeces of the replenished animals due to reduced absorption interfered with the absorption of P by reason of the formation of relatively insoluble calcium phosphates. The experiment does not, however, exclude the possibility that the neutralization of the HCl of the stomach did play a part in the high P excretion in the faeces of the animals on the high Ca diet in the preliminary period.

SUMMARY

1. The degree of Ca "saturation" of the tissues affects the efficiency of Ca utilization when Ca is furnished at levels below the requirement for maximum retention.

2. Under the conditions of these experiments the P excretion in the faeces tends to run parallel with the Ca excretion.

3. Lack of Ca in the diet affects the appetite adversely.

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CLXXII. DETERMINATION OF CALCIUM IN RAT URINE

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The assay of parathyroid hormone by the method of Dyer [1937] requires the mass determination of Ca in small amounts of rat's urine. The methods given in the literature apply chiefly to human urine, of which large amounts are readily available, and involve incineration, which greatly limits their usefulness for mass analysis.

The chief objection to precipitating Ca oxalate directly from urine appears to have been the danger of simultaneous adsorption or precipitation of uric acid [cf. McCrudden, 1911–12]. This is not very probable in the case of rat or mouse urine, in which uric acid is replaced largely by allantoin, and could probably be avoided in any case by varying the concentration and *p*H of urine and the duration and temperature of precipitation. In the case of rat or mouse urine a further disturbing factor enters, namely, the invariable presence of protein in these urines, and this has, as will be seen later, a small but definite interfering effect.

The object of the present research was to determine whether, and under what conditions, the microtitration method of Halverson & Bergeim [1917] is applicable to urine, deproteinized or not, the results being compared with those obtained for ashed urine.

Experimental

(1) *Materials*

Rat and mouse urines were collected in glass metabolism cages of the type described by Dyer [1937], with groups of 5 rats or 10 mice in each cage; the daily output was made up with diluted acetic acid to 100 ml. Human urine was taken from healthy adults.

(2) *Methods*

The Halverson-Bergeim technique, as modified by Van Slyke & Sendroy [1929], was applied in all cases to the final determination of Ca. The following minor modifications were found desirable. The precipitate often has a tendency to adhere to the sides of the tube and to float on the surface of the liquid. This can be obviated by adding oxalate to the solution at 80° and allowing it to cool to room temperature; large crystals of Ca oxalate are thus obtained which readily settle. Three washings with 1:50 ammonia are preferable to two, as recommended by Van Slyke.

The filtered acidified urines were deproteinized by mixing 10 ml. of 20% trichloroacetic acid with 25 ml. of diluted urine (containing 0·4–0·8 mg. Ca), and centrifuging after 30 min.

In view of the destructive action of phosphates on platinum vessels, dry incineration is not a desirable procedure. Wet ashing with H_2SO_4-$(NH_4)_2S_2O_8$, by the method of Shohl & Pedley [1922], or with H_2SO_4-$KHSO_4$ gave much lower results than those obtained by direct precipitation, both for urine and for standard $CaCl_2$ solution. Since the only difference in the final solutions was the presence of Na_2SO_4, this was added in amounts of 0–1 g. to 10 ml. of standard solution containing 0·1235 mg. Ca, and Ca was determined as above. The amount recovered fell rapidly as the amount of $SO_4^=$ added exceeded 0·1 g., being only 0·06 mg., or 50 % of the actual value, with 0·9 g. $SO_4^=$. It follows that microdetermination of Ca is not possible under the given conditions of incineration.

Ashing with HNO_3, by the method of Dyer, gave results which were substantially identical with the theoretical results. Thus the Ca content of a standard solution was 0·3169 mg. by direct precipitation, and 0·3189 mg. after incineration. Applied to urine the method also gave satisfactory results, and the values so obtained were taken as representing the true Ca content of the urines.

Apart from protein, uric acid has been supposed to interfere with direct precipitation of Ca. The precipitates obtained from amounts of urine (rat, mouse or human) containing 0·15–2 mg. Ca were tested by the Folin-Denis reaction for uric acid, but in no case was a positive reaction obtained, even after standing for 24 hr.

A necessary preliminary to the study of conditions of precipitation from urine was to determine the effect of varying the conditions under which Ca was determined in standard solutions, namely, duration, temperature and *p*H of the precipitation, time of centrifuging and number of washings. The results, given in Table I, show that the degree of accuracy is the same whether precipitation takes place at 3° or at 37°, and that precipitation is complete after 3 hr. Varying the *p*H from 3 to 6 does not significantly affect the results and the same is the case when the time of centrifuging is varied from 5 to 15 min.

Finally, it appears from the last column that the precipitates should be washed with three 3 ml. portions of 1 : 50 ammonia. It is evident that the

Table I. *Effect of varying duration, temperature and pH of precipitation, time of centrifuging and number of washings. All tubes contain 0·1235 mg. Ca. The figures in parentheses refer to the number of determinations, of which the mean is given*

Temperature at which Oxalate added	Systems kept before centrifuging	Mg. Ca found with various intervals elapsing before centrifuging: 40 min.	1 hr.	2 hr.	3 hr.	4 hr.	24 hr.	Time of centrifuging min. at 3000 r.p.m.	*p*H	No. of washings
19–21°	19–21°	0·1188 (5)	0·1196 (5)	0·1154 (3)	0·1188 (5)	0·1202 (3)	0·1181 (3)	15	4·5	3
19–21°	3°	0·1147 (2)	0·1198 (2)	—	0·1238 (2)	—	—	15	4·5	3
19–21°	37°	0·1188 (2)	0·1188 (2)	—	0·1268 (2)	—	—	15	4·5	3
80°	20°	—	—	—	—	0·1188 (2)	—	15	3	3
80°	20°	—	—	—	—	0·1233 (2)	—	15	3–4	3
80°	20°	—	—	—	—	0·1263 (4)	—	15	4–4·5	3
80°	20°	—	—	—	—	0·1239 (6)	—	15	4·5–5	3
80°	20°	—	—	—	—	0·1223 (2)	—	15	5–6	3
80°	20°	—	—	—	—	0·1256 (3)	—	5	4·5	3
80°	20°	—	—	—	—	0·1288 (2)	—	10	4·5	3
80°	20°	—	—	—	—	0·1225 (3)	—	15	4·5	3
80°	20°	—	—	—	—	0·1369 (2)	—	15	4·5	1
80°	20°	—	—	—	—	0·1268 (3)	—	15	4·5	2
80°	20°	—	—	—	—	0·1241 (3)	—	15	4·5	3

method is applicable over a wide range of conditions, which might be so selected as to exclude the interfering action of urinary constituents.

An analogous study was made of the optimum conditions for the determination of Ca in deproteinized rat urine. On the basis of these results the following method was adopted. The metabolism cage is rinsed with 5% acetic acid and the urine + washings are diluted to 100 ml. 10 ml. of 20% trichloroacetic acid are mixed with 25 ml. of filtered diluted urine and the solution is centrifuged after 30 min. (15 min. at 3000 r.p.m.). 5 ml. of the clear solution are diluted to 10 ml. in a calibrated conical centrifuge tube, and 1 ml. of 20% Na acetate is added, followed by 6 drops of bromocresol green indicator. 1 : 1 ammonia is then added drop by drop, with constant stirring, to a yellowish-green colour, indicating a *p*H of 4·5–5. The tubes are then placed in a water bath at 80°, and 1 ml. of 4% ammonium oxalate is added. After 5 min. the tubes are removed from the water bath, and are maintained at room temperature for < 4 hr., after which they are centrifuged (5 min. at 2500–3000 r.p.m.). The supernatant fluid is removed by suction, leaving 0·2 ml. of solution, the precipitate is dissolved in 2 ml. of N H_2SO_4 (5 min. at 100°) and the solution is titrated at 70° with 0·01 N $KMnO_4$.

Table II. *Effect of varying duration and temperature of precipitation in deproteinized rat urine*

Temp. before centrifuging	Mg. Ca found in 5 ml. urine, with a precipitation period of		
	1 hr.	2 hr.	3 hr.
3°	0·0714	0·0704	0·0734
21°	0·0765	0·0805	0·0754
37°	0·0775	0·0815	0·0805

The effect of varying the duration and temperature of precipitation is shown in Table II; the values given represent the means of two determinations. It appears that higher values are obtained at 37° than at 3°, but that with a duration of precipitation of 3 hr. the difference is not significant. Over a *p*H range of 3·5–6·5 (Table III) the highest values were obtained at *p*H 4·25–4·55. At *p*H 6·5 phosphate crystals (probably $MgNH_4PO_4$, $6H_2O$) were formed in some cases, and these interfered with the collection and washing of the Ca oxalate precipitate. As in the case of standard $CaCl_2$ solutions, 3 washings of the precipitate were found to be essential. Varying the duration of centrifuging from 5 to 15 min. did not affect the results. Ca was determined in 2–10 ml. of deproteinized urine; the results, calculated for 5 ml. of urine, were 0·1157, 0·1110, 0·1082, 0·1128, 0·1131 and 0·1107, for 2, 3, 4, 5, 8 and 10 ml. of urine respectively, corresponding with maximum deviations from the mean of $\pm 0{\cdot}012$ mg.; this difference does not exceed the maximum error of the titration.

Table III. *Completeness of precipitation of Ca from deproteinized rat urine at varying* p*H*

*p*H	3·55	3·75	4·25	4·40	4·55	5·90	6·50
Mg. Ca	0·0735	0·0754	0·0886	0·0835	0·0825	0·0815	0·0775

A comparison of the results obtained with and without deproteinization (Table IV) shows that whilst the mean results do not in any case differ by more than the permissible error of titration, yet the differences between the individual determinations are greater in presence of protein, and the mean results are consistently lower.

Table IV. *Results of determining Ca in untreated and deproteinized rat urines. All values refer to 5 ml. of urine*

No. of urine	Mg. Ca found in: Untreated urine		Deproteinized urine		Difference
1	0·087 0·080 0·077	0·081	0·092 0·088 0·088	0·089	0·008
2	0·118 0·110 0·112	0·113	0·118 0·117 0·124	0·119	0·006
3	0·166 0·160 0·153	0·159	0·163 0·165 0·165	0·164	0·005
4	0·086 0·087	0·087	0·098 0·102	0·100	0·013

A comparison of the results obtained for deproteinized and ashed rat urines (24 samples) gave a mean difference of 0·009 mg. in 5 ml. of urine in favour of the ashed urines. The difference is not significant, and it was hence concluded that incineration is not essential.

The results for mouse urine were not so satisfactory (Table V), as the differences between deproteinized and ashed urines in some cases exceeded 0·03 mg. In the case of human urines even greater differences were found, the values for ashed urine being from 0·015 to 0·060 mg. higher than for untreated urine. It may perhaps be concluded from these results that part of the Ca of human urine is not precipitable by oxalate and that under our conditions uric acid is not an interfering factor.

Table V. *Determination of Ca in mouse and human urines. The values given are the means of 3 determinations*

No. of urine	Source of urine	Mg. Ca found in 5 ml. of: Untreated urine *a*	Deproteinized urine *b*	Difference *a–b*	Ashed urine *c*	Difference *b–c*
1	Mouse	0·267	0·303	−0·036	0·270	+0·033
2	,,	0·272	0·265	+0·007	0·262	+0·003
3	,,	0·158	0·196	−0·038	0·189	+0·007
4	,,	0·235	0·247	−0·012	0·266	−0·019
5	,,	0·287	0·289	−0·002	0·307	−0·018
6	,,	0·262	0·280	−0·018	0·293	−0·013
7	,,	0·315	0·349	−0·034	0·374	−0·025
						a–c
1	Human	0·325	—	—	0·356	−0·031
2	,,	0·435	—	—	0·495	−0·060
3	,,	0·728	—	—	0·772	−0·044
4	,,	0·497	—	—	0·539	−0·042
5	,,	1·683	—	—	1·713	−0·030
6	,,	0·304	—	—	0·319	−0·015

SUMMARY

1. Calcium can be determined with satisfactory accuracy in small amounts of deproteinized rat urine by a modification of the method of Halverson & Bergeim. Incineration of the urine is not essential, but deproteinization is desirable.

2. Uric acid is not precipitated together with Ca oxalate, nor is it adsorbed by the precipitate. Higher values are given by incinerated than by untreated human urine.

3. Precipitation of Ca oxalate is inhibited by $SO_4^=$ in concentrations of over 1%. Methods of ashing involving the addition of H_2SO_4 are not applicable to the micro-determination of Ca in urine.

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CLXXIII. INVESTIGATIONS INTO THE METHOD OF ESTIMATING VITAMIN E

II. FURTHER OBSERVATIONS ON VITAMIN E DEFICIENCY AND IMPLANTATION

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The response to vitamin E treatment cannot be graded satisfactorily. Attempts to make use of the size of a litter, while ignoring the viability of its individual pups, would seem to have little justification; nor can the observer always be certain that he has in fact observed all the pups, dead or alive, that have actually been expelled from the maternal organism. The simplest procedure seems, therefore, to treat the response as of the all or nothing kind, and it becomes necessary to define what we mean by the birth of a litter.

We assess as a positive response the appearance of any number of animals, from one upwards and of whatever viability, whether they are found alive or not; it is clearly impossible to know in every instance whether all members of a litter were actually breathing at the time of birth.

We were at one time faced with a further difficulty. On the 22nd or 23rd day after mating it would sometimes be found impossible to tell whether a resorption had occurred and the response was accordingly to be judged negative, or whether a small number of non-viable young had indeed been born, only to be eaten by the mother before the cage had been inspected. In the absence of any sign of young, such a response, which should by definition have been judged positive, would have been recorded as negative. Accordingly, all cages containing does that have shown positive implantation are now fitted, on the 19th or 20th day after mating, with a movable grid, of mesh about $\frac{3}{4}$ in., placed $2\frac{1}{2}$ in. above the cage bottom. The doe remains on this grid, but the young that are born fall through on to the cage bottom. They seldom survive, but it is in any event our practice to destroy them; our sole concern is to have objective evidence that they have come into existence. Even so, a small number of animals may be consumed by the mothers before they have ever been deposited on the cage bottom (or grid); no account can be taken of these, whether the grid is used or not. The phrase "expelled from the maternal organism" must be read as meaning completely expelled and actually deposited by the mother on the floor of the cage.

We take as a measure of response what we call the fertility rate, or the percentage fertility, that is, the number of litters, as defined above, in relation to the number of positive implantations expressed as a percentage. The use of the fertility rate is exemplified later in this paper.

In an earlier publication [1937] we have advocated the use of virgin rats as test animals for vitamin E. Apart from the time saved by avoiding a preliminary gestation-resorption, a further economy is achieved in both time and material, as virgin animals show a higher implantation rate than those (R.P. animals) that have already undergone a gestation-resorption.

These results have been confirmed on a further large number of animals, drawn from our colony of highly inbred Wistar (London Strain) albino rats. The results are shown in Table I. In Table II we have combined the results from animals discussed in our earlier communication and those upon which Table I is based, but have re-divided them into 3 groups, according as the implanted animals produced no litters, up to one litter for every five implantations or more than this number. It will be seen from Table II that, at all 3 levels of vitamin E dosage, virgin animals showed much higher implantation rates than did the R.P. animals; the figures also confirm our former observation that the percentage of implantations is not affected by the level of vitamin E dosage after positive mating.

Table I. *Implantation rates*

Type of animal	No. of animals showing positive mating	No. of implantations	Implantation rate (%)
R.P.	59	32	54
Virgin	542	469	87

Table II. *Implantation rates*

	R.P. animals	Virgin animals
Dosage 0 (no litters)	61% (115)	90% (221)
Dosage ± (to 20% fertility rate)	74% (42)	90% (147)
Dosage + (>20% fertility rate)	56% (188)	87% (317)
Totals	59·7% (345)	88·6% (685)

The figures in parentheses indicate the numbers of animals mated.

In our previous paper we suggested as the most probable cause for the reduced implantation rate of R.P. animals that the process of gestation-resorption itself produces some permanent change in the animal's reproductive system. In order to test this point further, the following experiment has been carried out.

Twenty-five pairs of young female rats were placed at weaning on our basal diet, REES 1. When the animals came into oestrus, one of each pair was mated with a buck of known fertility, and mating was repeated if necessary until all 25 animals had shown the "plug" or the presence of live spermatozoa in the vaginal contents. Of these 25 animals, 24 showed the placental sign on the following 15th or 16th day; the 25th animal was re-mated and then became implanted. None of these 25 animals produced any living young, and all showed the symptoms of typical gestation-resorptions. They were all re-mated on showing oestrus again after the normal gestation period, and were then also given for 5 days a daily dose of 4·5 mg. of an extract prepared from wheat germ oil. Of these 25 R.P. animals, 15 showed failed implantation, giving the low implantation rate of 40%. Of the 10 implanted animals 5 had live litters, a "fertility rate" of 50%.

When the R.P. animals were about to be mated for the second time, their sisters were examined and also mated at the first normal oestrus. These were therefore virgin animals that had been submitted to the deficient diet REES 1 for some 3–4 weeks longer than is usual with our test animals, while they were waiting for their litter-mate sisters to be re-mated; their period on the diet, however, was identical with that of these R.P. litter-sisters. Of the 25 virgin animals, 21 showed positive implantations; this implantation rate of 84% is not far below the average for the whole of our recorded virgin animals. All 25 animals received the same dose of wheat germ oil extract as their R.P. sisters,

Table III. *Litter-mate comparisons*

Type of animal	Mated	Implanted	Fertile	Implantation rate %	Fertility rate %
R.P.	25	10	5	40	50
Virgin	25	21	16	84	76

and 16 of the 21 animals with positive implantations produced live litters, a fertility rate of 76%. The results are summarized in Table III.

It seems certain that in groups of 25 animals the difference between 40% implantation for R.P. animals, and 84% implantation for virgin animals, is highly significant; the differences in implantation rates cannot be due to different periods of vitamin E deficiency, since both groups of animals received the diet for the same time. It is probable, though not certain, that the difference in fertility rates of the 10 R.P. animals and the 21 virgin animals is also significant, indicating that the R.P. animals have not only a lower rate of implantation, compared with virgin animals, but also a higher vitamin E threshold. The relatively small number of R.P. animals involved in this comparison, however, makes it impossible for this point to be conclusively demonstrated.

We have some contributory evidence for this apparent difference in threshold. A sample of carefully preserved wheat germ oil concentrate (unsaponifiable matter largely freed from sterols by freezing) was in use for the preparation of a standard dose-response curve in experiments that are being made the subject of another communication. When tested on R.P. animals, this concentrate gave the following results:

Dose mg.	Implanted	Live litters	Fertility rate %
105	10	6	60
230	9	8	89

For the preparation of the dose-response curve only virgin animals were used. The comparable figures for fertility rates were:

Dose mg.	Implanted	Live litters	Fertility rate %
22·5	39	34	87·2
11·25	44	15	34·1

For both the higher and the lower doses of these two groups, it appears that R.P. animals may require 6 times as much vitamin E as virgin animals, if they are to show the same fertility rate. This threshold ratio is the highest we have encountered: it is more usually in the neighbourhood of 4 or 5 to 1.

In the classical monograph of Evans & Burr [1927] occur some observations on the relation between vitamin E and implantation. I am much indebted to Prof. J. C. Drummond for calling my attention to these remarks. Evans & Burr wrote: "We must call attention, firstly, to the fact that between five and eighteen per cent of all cases of 'positive' matings in normal animals, i.e. those held upon natural foods, result in failure to implant. Secondly, animals maintained on a basal ration and used to demonstrate the incidence of the specific sterility disease should not show an appreciably higher proportion of failed implantations than do animals maintained upon natural foods[1]. In our records of somewhat over fifteen hundred positive matings in animals on natural foods, only 82 to 95 per cent are followed by implantations. A much lower implantation per cent denotes either peculiar food inadequacy different in nature from that due to low E, or denotes the presence of some special toxic factor or general constitutional debility[2]." The footnotes [1] and [2] above also deserve quotation in full. "[1] The reader may naturally inquire as to whether

failure to implant may not possibly represent a more advanced stage of the particular sterility disease due to lack of vitamine E. It would appear that a conclusively negative answer can be given here inasmuch as, although we now have evidence of the gradual depletion of the body in its residual stores of E and the consequent necessity in old animals of somewhat higher curative doses of wheat germ oil, for instance, than is essential in young ones on the same diet, we do not have evidence of added difficulty in implantation in such cases. Furthermore, and more decisive—many cases of persistent failed implantation were given wheat germ oil in excess daily for over a month without effect on implantation." "[2] We would especially inform the reader in some groups of animals reared and held on the basal ration, the ovulation, acceptance of coition and especially the implantation have been so deficient as to demand the rejection of most individuals of the group. It is only when these phenomena are normal that animals can be used for experiments calculated to show the presence or absence of vitamine E in any dietary regime." The authors then go on to discuss the nature of food deficiencies and special toxic factors.

Whatever effect such factors may have on implantation rates, they have been as far as possible excluded in our experiments, for both groups of animals received treatments identical except in the one variable under examination. It is not clear whether the quoted remarks of Evans & Burr apply only to virgin animals or not; if they do, their observations are consistent with our own.

One possibility must be mentioned, namely, that it is the occurrence of gestation as such, and not the pathological process of a gestation-resorption, to which is to be attributed the reduced implantation rate. This, as we mentioned in our earlier publication [1937], is contrary to experience. For example, a very large colony of animals of the same strain as our own stock was maintained by the Macaulay Laboratory at the Institute of Animal Genetics in Edinburgh University for some years. Detailed records of mating are available for a considerable number of those animals [Wiesner, 1938]. They indicate that animals under 150 days old, mated for the first time, had a fertility rate of about 89 %. The animals were receiving a normal stock diet, and no question of vitamin E deficiency therefore arises: any failure in fertility of animals that had undergone positive mating must have been almost entirely due to failed implantation. The fertility of animals of similar age, positively mated after the birth of a litter, also showed about 89 % fertility, indicating no change in implantation rate.

In spite of this evidence, we have planned a further crucial experiment to distinguish between the effects on implantation rate of a "normal" gestation and the pathological condition of gestation-resorption. Meanwhile, we feel more than ever inclined to the view that toxic by-products from the resorbed embryos have inflicted some lasting damage on the uterine or other mechanism of implantation.

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CLXXIV. THE PHYSIOLOGICAL PROPERTIES OF ASCORBIC ACID

I. AN EFFECT UPON THE WEIGHTS OF GUINEA-PIGS

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The technique of paired feeding has been extensively used in nutritional investigations, particularly in those dealing with amino-acids and with vitamin B_1. The advantage of this procedure has been clearly indicated by Mitchell & Beadles [1930]. Its chief merit is that the otherwise variable factor of food consumption may be controlled. It has been known for some years that guinea-pigs, maintained on diets deficient in ascorbic acid, lose appetite, consume less food and diminish in weight. As in the case of vitamin B_1 it seemed reasonable that some of the symptoms manifest in these deficient animals might be caused by inanition. An extensive search of the literature has shown that only on one reported occasion has paired feeding been undertaken in studies on ascorbic acid. Anderson & Smith [1924], using a small number of animals and employing foods as sources of ascorbic acid, found that guinea-pigs fed a basal diet with supplements, the total amount being isocaloric with the consumption of scorbutic animals, were able to maintain body wt. at a higher level. The present investigation deals with an effect of *l*-ascorbic acid upon body wt. in paired feeding.

Methods

The basal diet, which was designed to be deficient only in ascorbic acid, was a modification of that used by Coward & Kassner [1936]. Its composition was as follows:

	%
Wheat bran	45
Crushed oats	25
Dried skim milk	25
Brewers' yeast	2
Cod liver oil	1
$CaCO_3$	1
NaCl	1

The dried milk was heated in open trays at 110° for 2 hr. with frequent stirring to destroy its content of ascorbic acid. This diet without supplement produced normal growth in young rats and, when supplemented with ascorbic acid, in young guinea-pigs.

Guinea-pigs reared in the Connaught Laboratories' colony, weighing 200–230 g., were used. They were housed in individual screen-bottom cages. Water was supplied *ad lib.* and food was given in detachable metal boxes deep enough to prevent spilling. The room temp. was regulated at 72–74° F.

For the first week of experimental feeding the basal diet was supplied *ad lib.* and each animal received daily oral doses of 5–10 mg. *l*-ascorbic acid in freshly prepared aqueous solution. Daily wt. records were kept. It was assumed that,

at the end of this preliminary week, all animals were saturated with ascorbic acid since daily doses of 2 mg. have been found to give maximal weight increase in this strain of guinea-pigs. This preliminary period also served to accustom the animals to changes in housing and diet. By the end of the week most animals showed a normal weight response; a few which failed to do so were discarded.

The animals were then divided into 3 groups, each individual of which corresponded as closely as possible to individuals in the other 2 groups. The division was made on the basis of wt., sex and rate of wt. increase during the preliminary week. The first group, designated as "normal", received the basal diet *ad lib.* and 2 mg. ascorbic acid daily. The second or "control" group was given the basal diet only. The third or "paired" group received 2 mg. ascorbic acid daily and an amount of food equivalent to that which had been consumed by the control group on the previous day. Animals in this group retained a good appetite throughout experimental feeding and ate all the food furnished. The animals were weighed daily after ascorbic acid was given to the normal and paired groups and before being fed. Daily records were kept of the amounts of food given to and consumed by each animal.

The duration of the experiment was 21 days after the preliminary week, the average survival time of the paired and control animals. If for any reason an animal in either the control or paired group died before this time, its wt. and those of the corresponding animals were not included in the averages which are reported.

Experimental results

Observations have been made on 8 series. In some of these there was no normal group. Concordant results have been secured throughout. Since results

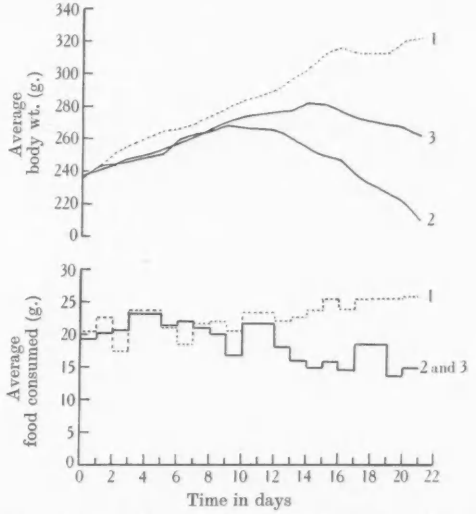

Fig. 1. Body weights and food consumption. 1, normal group; 2, control group; 3, paired group.

in good agreement have been given by all of 94 pairs in control and paired groups, data on one series only will be given. The accompanying figure shows

the curves of average wt. for three groups of 10 animals each and also shows the average food consumption for each group.

Discussion

Following a period of 10–12 days, during which body stores of ascorbic acid are presumably exhausted, control animals lose appetite and diminish in wt. until death occurs. When food is furnished *ad lib.* a continuous supply of ascorbic acid causes an increase in wt. This response has been used for the quantitative estimation of ascorbic acid by Bracewell *et al.* [1930], Harris & Ray [1932], Hou [1936] and by Coward & Kassner [1936]. Ascorbic acid prevents a loss in wt. in animals paired with the control group. At the end of 3 weeks there has been in every case a significant difference in wt. between animals receiving isocaloric intakes.

A loss of appetite in guinea-pigs deprived of ascorbic acid has been commented upon by other observers. Anorexia is as characteristic of a deficiency of ascorbic acid as it is of vitamin B_1 deficiency. The supply of ascorbic acid to the paired and normal groups prevented the loss of appetite. Appetite can be maintained by as little as 0·1 mg. ascorbic acid per guinea-pig per day.

There are several likely explanations of the difference in wt. between two animals given isocaloric intakes, one of which received ascorbic acid while the other did not. The difference might be due to alterations in water balance, in absorption of food, or in metabolism. Observations on these three factors are being conducted and will be reported later.

Summary

After 21 days of paired feeding young guinea-pigs receiving a scorbutic diet plus ascorbic acid are significantly heavier than animals of the same initial weight given only the basal diet. Since the difference in weight cannot be due to difference in food intake, it is believed to be due to alterations in metabolism, waterbalance and food absorption caused by a lack of ascorbic acid.

The authors are greatly indebted to the Banting Research Foundation for generous grants.

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CLXXV. ISOMERIZATION OF CAROTENOIDS

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It is of considerable interest to establish the limits within which the chromatographic method can be applied without danger of chemical alteration taking place in the column.

In the field of the carotenoids we were brought up against this problem [Zechmeister & Tuzson, 1938] in isolating the pigments of some fruits. The flesh of the water melon (*Cucumis citrullus*) in particular, which is rich in lycopene, sometimes gave extracts furnishing a single, very strong and homogeneous lycopene zone; in other experiments, however, carried out with the same raw material, the adsorbate showed a second layer below the lycopene ("*neo*lycopene"), which could be washed down by means of benzene and light petroleum more easily than lycopene and differed markedly in its brownish yellow colour from the reddish lycopene zone.

A closer examination of the discrepancy between our various experiments showed that the character of the chromatogram depended on the time which elapsed between the extraction of the tissues and the beginning of the Tswett analysis. Fresh solutions furnished a single lycopene layer, older ones two coloured zones on the same part of the column; analogous results were obtained with solutions of pure crystalline lycopene. Light was thrown on these experiments by the fact that a freshly prepared solution of lycopene obviously changes its optical properties when kept. Further, this spontaneous alteration can actually be measured by means of colorimetric and spectroscopic determinations. The Tswett technique thus only furnishes a convenient demonstration of an effect already present in the solution.

The electronic configuration of the chromophore contained in the lycopene molecule becomes much less stable when the compound is dissolved. If the solution is kept at room temperature it can be easily shown that the colour intensity gradually decreases and that the extinction maxima slowly wander towards the shorter wave-lengths. Such changes must have been frequently observed by earlier workers but were probably ascribed to autoxidation. It cannot indeed be denied that simultaneous oxidative reactions do take place in the solution even under practically anaerobic conditions.

We find that autoxidation of lycopene leads partly to a colourless material and partly to coloured substances which are readily adsorbed on the highest section of the column and which cannot be reconverted into lycopene. On the other hand, *neo*lycopene is always adsorbed below the lycopene zone and partly reverts to lycopene, if its zone is cut out and the elutriated substance is kept in a suitable solvent. The following experiments illustrate the processes indicated.

(*a*) A solution of 1·54 mg. lycopene (chromatographically homogeneous) in 20 ml. benzene was kept for 24 hr., diluted with light petroleum and chromatographed on $Ca(OH)_2$. Two layers were obtained, containing 1·26 mg. lycopene and 0·13 mg. *neo*lycopene. A similar experiment gave after 3 days 1·02 and 0·31 mg. respectively.

(*b*) 1·57 mg. pure *neo*lycopene in 10 ml. benzene gave after 4 hr. 0·05 mg. lycopene and 1·49 mg. *neo*lycopene; after 24 hr. 0·08 and 1·15 mg. and after 2 days 0·12 and 1·13 mg.

If the solution is heated, equilibrium between lycopene and its isomeride is reached in a much shorter time. As can be seen from Table I, 5 min. boiling under reflux is sufficient to cause noticeable effects and within 30 min. about half the lycopene undergoes rearrangement in benzene. A very similar result is obtained by heating *neo*lycopene in benzene (Table III).[1]

In the course of extended experiments we came to the conclusion that many former authors must have actually worked with solutions which had come to equilibrium in the course of the usual laboratory operations such as concentration, heating etc. Since *neo*lycopene is transformed into ordinary lycopene by crystallization, it is understandable that its very existence has been overlooked. We may also mention that in some earlier experiments carried out by Dr Cholnoky in this laboratory, delay in the crystallization of and decrease in the colour intensity of certain lycopene solutions were observed, which could not at that time be explained.

Isomerization reactions among carotenes were first detected by Gillam & El Ridi [1935] and thoroughly described in two subsequent papers [Gillam & El Ridi, 1936; Gillam *et al.* 1937]. In the course of this extended and interesting study *pseudo*-α-carotene and *neo*-α-carotene were isolated in pure state. The formation of these new compounds was explained by the action of the adsorbent on carotenes. In such way repeated adsorptions must lead to increasing quantities of the isomeride. Without belittling the importance of the work of Gillam *et al.* we maintain that the isomerization of β-carotene is in all probability as spontaneous as that of lycopene. We claim that in no case is the phenomenon caused by the adsorption process and we are not able therefore to agree with the statement that "the analytical process itself affects the substances which it is designed only to separate".

In view of the earlier work of Gillam & El Ridi, our experiments with β-carotene were confined to the attempt to obtain sufficient information concerning the possible role of the adsorbent. As may be seen from the figures given in Table IV, the isomerization of β-carotene is directed by the same factors as the rearrangement of lycopene, though in the former case the reaction proceeds much more slowly. We find calcium hydroxide to be the most suitable adsorbent for such investigations, but since Gillam & El Ridi generally used alumina, a series of experiments was carried out with this adsorbent. Using the materials available to us we find that if a sample of β-carotene has once reached chromatographic homogeneity, little or no *pseudo*-α-carotene appears when the adsorption is subsequently repeated, provided that no considerable time elapses between each experiment and further, that all elutriations etc. are carried out not far from room temperature. Under these conditions we are unable to confirm the statement of Gillam & El Ridi that the amount of the newly formed isomeride increases with successive adsorptions.

Similar observations were made with various solutions of kryptoxanthin (Tables V–VI).

Finally we may briefly discuss the question of the natural occurrence of the new isomerides. Working under the established conditions, it was observed that rapidly prepared and chromatographed extracts (*ex* tomatoes, water melon, berries of *Tamus communis* etc.) yielded perfectly homogeneous lycopene zones.

[1] Preliminary experiments seem to show that both isomerides are much more rapidly interconverted at room temperature in the presence of a small quantity of iodine.

*neo*Lycopene was only formed if the same extracts were kept for some time. Already after 2 hr. at 20° a small amount of *neo*lycopene could be demonstrated in the column. The red fruits of *Actinophleus Macarthurii* (from Java) which were conserved in ammonium sulphate for 2 years, gave only a trace of *neo*-lycopene. It is obvious that in these cases lycopene occurs in the tissue under conditions which inhibit isomerization and which from this point of view may be compared with the crystalline state.

Gillam & El Ridi [1936] claim that the isomerization of β-carotene is accelerated by the presence of some impurities: "Thus the carotene fractions of butter or blood serum...are separated readily into two zones of pigments by a single adsorption...whilst with pure crystalline β-carotene it requires much longer washing or more usually elution followed by another adsorption to bring about the isomerization." We suggest that these observations could well be explained either by the original presence of *pseudo*-α-carotene in the butter and blood serum pigment or by formation of it during the lengthy course of the preparation.

Experimental

(Partly in collaboration with Miss I. Berger)

Lycopene

Benzene solutions of crystalline lycopene (from various sources) were diluted with light petroleum (b.p. 60–80°), poured through a column of calcium hydroxide (25 × 5 cm.) and developed with a mixture of benzene and light petroleum 3 : 1. The main zone was cut out and eluted with benzene-alcohol. The latter was washed out and the remaining solution dried with Na_2SO_4 and evaporated rapidly *in vacuo* (bath temperature 40–45°). Under such conditions no isomerization takes place as was proved in special experiments. Working with small quantities the operation takes only 5 to 10 min.

The residue was dissolved in benzene, chloroform or acetone. If light petroleum were to be used in subsequent boiling experiments, the above evaporation could be omitted, the benzene being eliminated from the column by washing with sufficient quantities of light petroleum; the lycopene zone was then eluted with petroleum plus alcohol and the washed and dried eluate used directly for the boiling. Before proceeding to the latter an aliquot part of the solution was again chromatographed in order to check its absolute homogeneity. 20 ml. of each solution were boiled under reflux in an all-glass apparatus and the decrease of the colour intensity established by comparing directly the heated and the original solutions in a Leitz micro-colorimeter. The absorption maxima were measured with a Zeiss spectroscope.

Subsequently the cooled solutions were diluted with light petroleum and introduced into a calcium hydroxide column. (If acetone had been used, it was first eliminated by washing the solution.) In each case the chromatogram was developed with benzene and light petroleum and washed with a liberal quantity of the latter. A very good separation into two main zones was obtained, these being accompanied by much smaller layers on the upper part of the adsorbent.

The lycopene and *neo*lycopene zones obtained after 30 min. boiling were eluted with light petroleum-alcohol and, after elimination of the alcohol, compared with the Kuhn & Brockmann [1932] standard in the micro-colorimeter. As the tints of the two isomerides are different (lycopene reddish, *neo*lycopene brownish) it is advisable to use the solution of 145 mg. azobenzene in 100 ml. 96% alcohol for lycopene and a 10-fold dilution thereof for *neo*lycopene. The results are summarized in Table I.

Table I. *Partial conversion of lycopene into* neo*lycopene*

Solvent	mg. lycopene in 10 ml. of the original solution	Time of boiling min.	Spectrum mμ	Colorimetric value in % of the original	Amount of *neo*lycopene formed in % of the total pigment in the column
Benzene	0·68	0	522, 487	100	
,,	0·68	5	519, 484	87	
,,	0·68	15	518, 485	87	
,,	0·68	30	519, 485	82	55
,,	0·69	0	522, 487	100	
,,	0·69	5	519, 485	91	
,,	0·69	15	519, 484	84	
,,	0·69	30	519, 484	81	50
,,	1·25*	0	521, 485	100	
,,	1·25	30	519·5, 484	89	22
Chloroform	0·72	0	518, 484	100	
,,	0·72	5	517, 483	89·5	
,,	0·72	15	517, 482	87	
,,	0·72	30	516·5, 483·5	76	39
,,	0·82	0	518, 484	100	
,,	0·82	5	517·5, 484	86	
,,	0·82	15	517·5, 483·5	78·5	
,,	0·82	30	517·5, 482·5	73	36
Acetone	0·40	0	505·5, 475	100	
,,	0·40	5	505, 474	95·5	
,,	0·40	15	504·5, 473	90·5	
,,	0·40	30	504·5, 473	89·5	18
,,	0·54	0	505·5, 475	100	
,,	0·54	5	505, 474	96·5	
,,	0·54	15	504·5, 473	90	
,,	0·54	30	504, 473	83·5	20
Light petroleum	0·40	0	504·5, 472·5	100	
,,	0·40	30	502·5, 470	86·5	44
,,	0·28	0	504, 472	100	
,,	0·28	30	502, 470·5	85·5	42

* This solution was not boiled but kept at 56°.

neo*Lycopene*

This compound is much more easily soluble than lycopene itself and could not be obtained in crystalline form. Attempts to crystallize it from carbon disulphide-alcohol or benzene-methanol always led to samples which showed no perceptible difference from ordinary lycopene. The absorption bands of *neo*-lycopene show a displacement towards shorter wave-lengths (Table II).

Table II. *Absorption maxima of lycopene and* neo*lycopene* (*mμ*)

Solvent	Lycopene	*neo*Lycopene
Carbon disulphide	547, 505, 475	536, 498, 466
Benzene	522, 486, 445	512, 479, 450
Chloroform	516, 479·5, 434·5	512, 478, 447·5
Acetone	506, 475, 446	499·5, 468, 439
Light petroleum	506, 474, 445	499·5, 468, 439
Alcohol	504·5, 472·5, 445·5	500, 469, 439
Ether	503·5, 471·5, 443·5	496, 466, 438
Methanol	500, 470, 441	497, 467, 436·5

On heating *neo*lycopene solutions, the colorimetric value of the liquid increases with considerable velocity (Table III) and the polyene which at first shows no evidence of heterogeneity separates into two well-defined zones on

Table III. *Partial conversion of* neo*lycopene into lycopene*

Solvent	mg. *neo*lycopene in 10 ml. of the original solution	Time of boiling min.	Spectrum mμ	Colorimetric value in % of the original	Amount of lycopene formed in % of the total pigment in the column
Benzene	~ 1	0	513·5, 580	100	
,,	~ 1	15	518·5, 487	135·5	
,,	~ 1	30	519·5, 484·5	153	~ 42

calcium hydroxide. The chromatogram is typical for the equilibrium mixture which may be obtained by starting from ordinary lycopene under the same conditions; the lycopene zone yielded a sample which gave no depression of M.P. when mixed with pure lycopene (*ex* tomatoes).

For the purposes of this work it was necessary to have a rough estimate of the colorimetric value of *neo*lycopene, i.e. the amount had to be established which, when present in 1 ml. light petroleum, shows the same intensity as 14·5 mg. azobenzene in 100 ml. alcohol. Since *neo*lycopene has not hitherto been obtained crystalline, the chromatographically homogeneous and colorimetrically matched solution was evaporated to dryness. The *neo*lycopene content was about 0·003 mg. 1 ml., showing that the compound is much less coloured than ordinary lycopene, for which the corresponding figure is 0·002 mg./ml.

β-Carotene

The isomerization of this polyene takes place more slowly than that of lycopene. A solution of 50 mg. pure substance in 200 ml. light petroleum was kept for 24 hr. at room temperature and showed, on a calcium hydroxide or alumina column which was washed with the above solvent, only a slight zone of *pseudo*-α-carotene, the amount of which was about 1–2 % of the total carotene. The boiling experiments which are summarized in Table IV were carried out as

Table IV. *Partial conversion of β-carotene into* pseudo-α*-carotene*

Solvent	mg. β-carotene in 10 ml. of the original solution	Time of boiling min.	Spectrum mμ	Colorimetric value in % of the original	Amount of *pseudo*-α-carotene formed in % of the total pigment in the column
Benzene	1·04	0	496, 464	100	
,,	1·04	5	496, 464	100	
,,	1·04	15	494·5, 462·5	90	
,,	1·04	30	495, 463	88	18
,,	1·43	0	496·5, 465	100	
,,	1·43	5	496, 464	97	
,,	1·43	15	495, 463	92	
,,	1·43	30	495·5, 462·5	92	21
Chloroform	0·86	0	495, 462	100	
,,	0·86	5	495, 462	98	
,,	0·86	30	494·5, 461·5	95·5	
,,	0·86	60	494, 461·5	93	11
,,	0·90	0	495, 462	100	
,,	0·90	5	494·5, 461·5	98	
,,	0·90	30	494·5, 462	97	
,,	0·90	60	494·5, 461·5	97	12
Acetone	1·33	0	486, 455	100	
,,	1·33	30	485·5, 453	~ 100	~ 0

described above, with the sole difference that the chromatogram was developed by means of pure light petroleum.

In a fairly long series of experiments we tried to establish whether the quantity of the isomeride increases or not from one adsorption to the next; the following data are typical.

(*a*) A solution of 100 mg. pure β-carotene in 150 ml. light petroleum (B.P. 60–80°) was chromatographed on alumina (mixture of 3 parts of Al_2O_3 Merck and 1 part standardized alumina, 25 × 5·7 cm.) and the column washed first with 1 l. light petroleum and then with 4 l. light petroleum plus 5 % benzene. Traces of by-products fixed on the upper part of the column, as also a very small amount of *pseudo*-α-carotene, were neglected. We eluted the β-carotene zone with light petroleum-alcohol. Then the liquid was washed, dried and poured through a new column. The *pseudo*-α-carotene layer of this second chromatogram was much smaller than before, and it disappeared totally when a third chromatographic analysis was rapidly carried out.

(*b*) An analogous experiment with calcium hydroxide gave us a small *pseudo*-α-carotene zone. In this case, however, even the second chromatogram was composed exclusively of a single carotene layer and in spite of abundant washing no isomeride appeared.

Kryptoxanthin

As may be seen from Table V, this polyene-alcohol exhibits a tendency to spontaneous isomerization which, under similar conditions, is greater than that of β-carotene. Solutions of *neo*kryptoxanthin may easily be converted into the

Table V. *Partial conversion of kryptoxanthin into* neo*kryptoxanthin*

Solvent	mg. kryptoxanthin in 10 ml. of the original solution	Time of boiling min.	Spectrum mμ	Colorimetric value in % of the original	Amount of *neo*kryptoxanthin formed in % of the total pigment in the column
Benzene	0·86	0	496, 463	100	
,,	0·86	5	495, 462	96·5	
,,	0·86	15	494·5, 461	92	
,,	0·86	30	493·5, 461	90	28
,,	2·04	0	496, 463	100	
,,	2·04	5	495, 462·5	97	
,,	2·04	15	494, 462	91	
,,	2·04	30	493·5, 461·5	88·5	30
Chloroform	0·97	0	494, 462	100	
,,	0·97	5	493·5, 461	95·5	
,,	0·97	15	493, 461	95·5	
,,	0·97	30	493, 461	94	21·5
Acetone	1·14	0	487, 455	100	
,,	1·14	30	485, 454	92	15

Table VI. *Partial conversion of* neo*kryptoxanthin into kryptoxanthin*

Solvent	mg. *neo*kryptoxanthin in 10 ml. of the original solution	Time of boiling min.	Spectrum mμ	Colorimetric value in % of the original	Amount of kryptoxanthin formed in % of the total pigment in the column
Benzene	~ 1	0	491, 460	100	
,,	~ 1	5	492, 461	103	
,,	~ 1	15	493, 461	106·5	
	~ 1	30	493, 461	110	46·5

equilibrium mixture with kryptoxanthin (Table VI). The absorption maxima of pure *neo*kryptoxanthin solutions are given in Table VII.

Table VII. *Absorption maxima of kryptoxanthin and* neo*kryptoxanthin* ($m\mu$)

Solvent	Kryptoxanthin	*neo*Kryptoxanthin
Carbon disulphide	518, 482	510, 478
Benzene	497, 464	490·5, 459
Chloroform	494, 462·5	490·5, 458
Acetone	486, 454	480·5, 449
Light petroleum	484·5, 451·5	478, 447
Alcohol	484, 452	479, 448

SUMMARY

1. Solutions of chromatographically pure lycopene $C_{40}H_{56}$, β-carotene $C_{40}H_{56}$ or kryptoxanthin $C_{40}H_{56}O$ undergo, when kept at room temperature, a partial isomerization which manifests itself in the decrease of the colorimetric value and in the displacement of the absorption maxima towards shorter wavelengths.

2. The speed of this spontaneous isomerization, which tends towards an equilibrium, increases on heating.

3. The interconversion is reversible.

4. Partly isomerized solutions always give two distinct layers in the Tswett column; the phenomenon is not caused by the adsorption experiment itself, but is already present in the solution. Experiments now in progress might show how far *cistrans* changes are responsible for it [cf. Gillam *et al.* 1937].

Finally we wish to thank Dr A. E. Gillam for having kindly checked some of our experiments with lycopene. This work was aided by grants from the Duke of Eszterházy and from the Széchenyi Society.

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CLXXVI. COLORIMETRIC DETERMINATION OF SUBSTANCES CONTAINING THE GROUPING $-CH_2.CO-$ IN URINE EXTRACTS AS AN INDICATION OF ANDROGEN CONTENT

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In the expectation that a measure of endocrine activity in the human patient might be given by the level of excretion in the urine of compounds related to the male hormones, attention has recently been directed to the extraction of urinary constituents possessing androgenic activity and their measurement. As an alternative to biological assay, chemical methods have been applied. Zimmermann [1935] first suggested that steroid sex hormones could be determined quantitatively by the use of *m*-dinitrobenzene, which gives a red colour in presence of alkali with compounds containing an active methylene group. A year later [Zimmermann, 1936] he published a modified method which was applied to pure compounds (androsterone, testosterone, oestrone and equilin), and to urine extracts. Wu & Chou [1937] described a modification of the method, and used it on urine extracts with androsterone as a reference substance. Oesting & Webster [1938; cf. Oesting, 1937] used the Zimmermann technique, and roughly correlated their figures with the comb-growth produced in capons by inunction of the same extracts on the comb.

None of these investigators has considered simultaneously both capon assay and colorimetric assay in terms of pure hormones, and there was thus an obvious gap to be filled before it would be possible to test the colorimetric assay as an indicator of the androgenic activity of urine extract in terms of international units. The correlation of the figures obtained in this way would be expected to give data which, in addition to testing the value of the colorimetric assay on the assumption that excretion of androgenic activity was diagnostically significant (as has been done by Oesting & Webster), would also throw light on the chemical nature of the androgenic substances excreted in the urine, providing clues to the metabolism of steroid hormones, and giving a tangible chemical property which would serve as a guide in the analytical investigation of urine extracts.

As part of a scheme of work on the determination of hormones in blood and urine, undertaken under the auspices of the Hormones Committee of the Medical Research Council, investigations of methods of extraction of androgens from urine and their assay on capons have been in progress at this Institute for some time. These have been extended to a comparison of colorimetric and capon assays, and, on the chemical side in particular, to attempts to improve the sensitivity of the colorimetric method and to investigate its specificity.

The account of experimental work which follows deals in turn with the description of the modified colorimetric method finally adopted, a study of some of the many factors influencing the reaction, an investigation of the behaviour

of various methyleneketones of steroid and other types, and the correlation of colorimetric assay with capon assay for urine extracts from normal and certain abnormal clinical types.

Experimental

Reagents. (1) *Routine method of colorimetry of androsterone*

(*a*) *Alcohol.* Ordinary commercial "absolute" alcohol is used, the only further specification being that it should not have a content of aldehyde exceeding 0·0025 %.

(*b*) m-*Dinitrobenzene.* A well-crystallized and fairly pure material (we used B.D.H. "extra pure", M.P. 89–89·5°) is taken and further purified as follows: 20 g. are dissolved in 750 ml. of 95 % alcohol warmed to 40° and 100 ml. of 2*N* NaOH are added. After 5 min. the solution is cooled, and 2500 ml. of water are added. The precipitated *m*-dinitrobenzene is collected on a Büchner funnel, washed very thoroughly with water, sucked dry and recrystallized twice in succession from 120 ml. and 80 ml. of absolute alcohol. The material must be well crystallized in almost colourless needles, M.P. 90·5–91°. Admixture of a 1 % alcoholic solution with an equal vol. of aqueous 2*N* NaOH should give no colour after an hour. The reagent is a 2 % w/v solution of this material in absolute alcohol. It is stored in a brown, stoppered bottle in the dark, and is stable for 10–14 days. In the actual colorimetric measurement (see below) the control solution, without methyleneketone, should give a pale straw colour having a value of $E_g = 0{\cdot}20$–$0{\cdot}21$ in a 1 cm. cell compared with alcohol.

(*c*) *Potassium hydroxide.* The reagent solution is 2·5*N* KOH in absolute alcohol. 9 g. of KOH (B.D.H. "Analar" pellets) are dissolved with mechanical stirring in 50 ml. of absolute alcohol, and the solution filtered through a hardened paper (Whatman No. 50) at the pump. The concentration is checked by titration of 0·5 ml. with 0·1*N* H_2SO_4 (methyl orange indicator) and the solution diluted with alcohol if necessary, to bring it within the limits of 2·48 and 2·52*N*. The solution is stable for 2–5 days if stored in a refrigerator. It must be discarded as soon as the faintest colour is perceptible.

Mode of operation

Test tubes used for the reaction must have been cleaned with nitric and chromic acid mixture. Into one tube, to serve as "blank", are measured out in succession, from 1 ml. pipettes graduated to 0·01 ml., 0·2 ml. of alcohol, 0·2 ml. of *m*-dinitrobenzene solution and 0·2 ml. of KOH solution. The solution of the test substance is measured out into a second tube, and then there is added sufficient alcohol to make the volume up to 0·2 ml., followed by the reagents. The time of adding the KOH is noted. The tubes are well shaken to disperse the dense KOH solution, lightly stoppered, and placed in a water bath kept at $25 \pm 0{\cdot}1°$ by means of a thermo-regulator. The tubes are shielded from all but dull, diffused light by a screen. After an hour, 10 ml. of alcohol are added to each tube, and the contents mixed and transferred to the cells of the colorimeter, which are then closed by microscope coverslips.

Measurement of the colour

Use of the Spekker photoelectric absorptiometer. In this instrument the light absorbed by a coloured solution is measured photoelectrically by a null method. The light transmitted by the test solution falls on a photoelectric cell, the current from which is balanced by current from a second cell with an adjustable iris in front of it. The test solution is then replaced by the blank or control

solution, and balance again obtained by closing in the path of the beam a shutter actuated by a wheel with a logarithmic scale, from which an "absorption coefficient" (E) is read off directly. Using light which has passed through a selective filter, a value of E for a limited range of wave-lengths is obtained. In this work, cells of 1 cm. thickness have been employed, with an Ilford "spectrum green" filter (max. transmission at 5200 Å.). As a routine, readings (denoted by E_g) on one pair of solutions are made in triplicate: in our experiments these differ by not more than 0·01 over the range $E_g = 0{\cdot}1$–$0{\cdot}5$, which is the most suitable for measurement. It is desirable to complete the measurement within 5 min. of the dilution with alcohol. The methyleneketone content of the test substance in terms of androsterone is then determined from the absorptiometer reading (E_g) by reference to a calibration curve constructed from measurements with androsterone. All the recorded measurements, unless otherwise specifically stated, have been made by comparison of the test solution with a control blank containing reagents only.

Calibration curve. The androsterone used as standard was a purified sample with M.P. 184–185° (corr.). In the beginning of this work measured amounts of an alcoholic solution were evaporated to dryness in a stream of nitrogen on a

Table I. *Calibration of Spekker absorptiometer*

Androsterone (mg.)	0·025	0·05	0·075	0·10	0·15	0·20	0·25	0·30
E_g (*a*)	—	0·205	—	0·395	0·545	0·66	0·74	0·80
E_g (*b*)	0·10	0·19	0·275	0·365	0·50	0·60	—	—

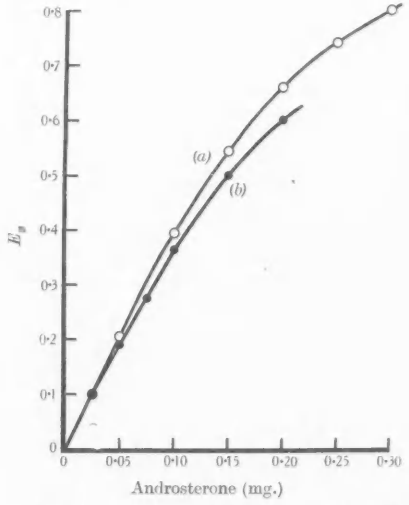

Fig. 1. Calibration curves for the reaction: androsterone + m-$C_6H_4(NO_2)_2$ + KOH (test solution – blank) on Hilger Spekker absorptiometer with Ilford "spectrum green" filters.

water bath, and alcohol and reagents added to the residue. It was soon found that loss occurred owing to the volatility of androsterone, and after this the androsterone was measured out in the form of appropriate amounts (0·1–0·2 ml.) of 0·025–0·1 % solutions in absolute alcohol, made up to a total volume of 0·2 ml.

Using the precautions specified above, it has been possible to reproduce colorimetric values for certain weights of androsterone within comparatively narrow limits, but there is a residue of variation which we have not been able to control. In all probability this is due to impurities in the alcohol and the *m*-dinitrobenzene. In view of the number of factors influencing the reaction, as described in detail below, the agreement is satisfactory. Over a period of seven months, during which the calibration curve has been frequently checked, the maximum variation has been between the limits shown in Table I and curves (*a*) and (*b*) in Fig. 1.

At the upper limit suggested for readings, $E_g = 0{\cdot}5$, the difference, in terms of weight of androsterone, is 12%. This difference does not, of course, represent the error in the determination of unknown materials when the position of the calibration curve with a new set of reagents is confirmed in the routine manner by colorimetry of a known amount of androsterone. Thus, ten measurements with 0·1 mg. of androsterone over a period of 12 days with two apparently similar batches of reagents gave a value of 2·0% for the standard error of the absorptiometer reading, corresponding to a standard error of 2·7% in the estimation of the weight of androsterone after reference to the calibration curve.

Use of a visual colorimeter of plunger type. With the co-operation of Mr S. W. Stroud, working in the Courtauld Institute of Biochemistry, Middlesex Hospital, an investigation has been made of the use of a Leitz two-stage colorimeter in the colorimetry of androsterone and urine extracts. With this instrument observations can be made with light which has passed through selective filters. It is only necessary to choose a colour standard. Potassium permanganate solution has an absorption spectrum which is very similar in the green, except for its banded character, to the net absorption spectrum of androsterone and reagents less that of reagents alone. Permanganate solution plus blank can therefore be matched approximately against test solution plus water, and accurate matching is possible in the light transmitted by the Leitz "yellow-green, 531" or "green-yellow, 551" filters: the former, transmitting light nearer the centre of the absorption band, gives higher readings.

The colorimeter is set up with test solution, prepared in the usual way, in the top cup and water in the bottom cup on one side, and, on the other side, blank in the top cup and $N/1000$ $KMnO_4$ in the bottom cup, the last being in a 20 mm. layer. The top plungers, which move together, are adjusted until the beams through the two sides are matched, and the depth of the layer is then read off on the scale.

A calibration curve was constructed with weights of androsterone from 0·05 to 0·225 mg. and the data are given in Table II and plotted in Fig. 2 in the form of weight of androsterone against the reciprocal of the scale reading.

Estimations made in this way on six urine extracts were compared with estimations on the Spekker absorptiometer, and gave the following results in terms of colorimetric equivalent, in mg., of androsterone per day's output, the pairs of figures being from Leitz and Spekker instruments, respectively: (*a*) 7·5, 8·0; (*b*) 7·0, 7·5; (*c*) 15·5, 15·3; (*d*) 15·7, 15·8; (*e*) 1·5, 1·75; (*f*) 24·0, 22·5. The agreement is fairly good. The disadvantage of this technique with the Leitz instrument is the impossibility of making absolute determinations of the absorption in the violet. As explained below, this may be an important matter in the recognition of occasional urine extracts in which the chromogen is abnormal, and the value of E_g alone bears no relation to the maximum of the absorption band.

Table II. *Calibration of Leitz colorimeter*

Androsterone (mg.)	0·05	0·075	0·10	0·125	0·15	0·175	0·20	0·225
Scale reading (531 filter)	38·3	23·4	17·1	14·3	11·9	10·0	8·7	8·4
Scale reading (551 filter)	31·5	19·3	14·6	11·3	9·2	7·6	7·3	6·6

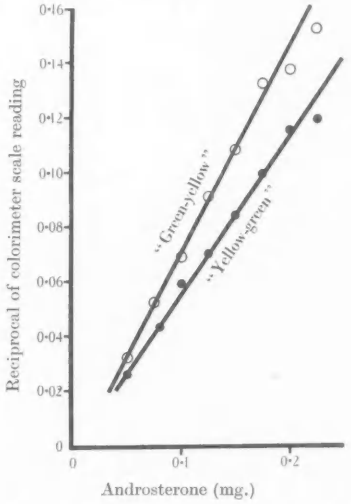

Fig. 2. Calibration curves for the reaction: androsterone + m-$C_6H_4(NO_2)_2$ + KOH (test solution + water/blank + 0·001N $KMnO_4$) on Leitz colorimeter with "green-yellow" and "yellow-green" filters.

Application to urine extracts

Colorimetric measurements have been made on the neutral fractions of urine extracts made by the method described by one of us [Callow, 1936, 1] with minor modifications. The acid and phenolic fractions contain ketonic material, and must always be removed. The neutral fraction is usually taken up in alcohol at a concentration such that 1 ml. contains the equivalent of either 0·1 l. of original urine, or 0·1 day's output, and 0·1 ml. of this solution is taken and diluted with 0·1 ml. of alcohol for colorimetry as described above. The colour of the urine extract itself is not sufficient to interfere with the determination. An unusually highly coloured extract gave a value of $E_g = 0{\cdot}01$ when diluted in the proportion of 0·01 l. urine to 10·6 ml. Moreover, treatment with charcoal generally produces no appreciable change in the colorimetric reading (Oesting [1937] found that androgenic activity was not lost in this way). As in the case of androsterone, evaporation of alcoholic solutions of urine extracts, unless very carefully done, entails loss not only by volatilization but also by creeping, and this method of taking a known amount was abandoned.

The absorptiometer reading is converted, by reference to the calibration curve, into an equivalent in weight of androsterone, which is then expressed as mg. of "sterone" per litre or per day's output of urine. We have coined the term "sterone", to avoid giving the wrong impression that this value represents the biological and not merely the chromogenic equivalent of androsterone.

(2) *Factors influencing the reaction with androsterone*

General. In preliminary trials it was immediately found that, with unpurified *m*-dinitrobenzene, the technique of Wu & Chou [1937] gave a higher colorimetric reading than that of Zimmermann [1936] with the same amount of androsterone; the former method was therefore chosen as the starting-point for investigation of the factors influencing the sensitivity. Certain of these had already been studied by Zimmermann (Z.) and by Wu & Chou (W.C.), namely: (1) the solvent (Z., W.C.), (2) concentration of alkali (Z., W.C.), (3) concentration of *m*-dinitrobenzene (Z., W.C.), (4) time of measurement after mixing (Z.), (5) temperature of development (Z., W.C.), (6) illumination (Z.). With so many variables concerned (and this list is not exhaustive) it was to be expected that further systematic work would lead to an increase in the sensitivity of the reaction. Moreover, by choosing conditions under which the effect of variation in any factor was changing most slowly, the reproducibility of the readings might be increased.

Solvent: purity and amount. Small amounts of aldehyde in the alcohol cause increased absorption in both the test and the blank solutions, but more in the latter, so that, as a result of over-compensation, the value of test minus blank decreases with increase in the aldehyde. The figures in Table III were obtained using our routine technique with 0·1 mg. of androsterone. The absorptiometer with the "spectrum yellow-green" filter was used for the determination of aldehyde in samples of alcohol by means of the colour produced with Schiff's reagent. In separate experiments the same alcohol, of known aldehyde content, was used for dissolving the androsterone, the *m*-dinitrobenzene and the potassium hydroxide.

Table III. *Influence of acetaldehyde on* E_g *with 0·1 mg. androsterone*

% MeCHO	Test minus blank	Blank minus EtOH	Test minus EtOH (calc.)
0·0005	0·395	0·11	0·505
0·0017	0·37	0·17	0·54
0·0025	0·35	0·195	0·545
0·005	0·31	0·27	0·58

The effect of altering the total amount of solvent in the reaction mixture was tested as follows: portions of 0·25 mg. of androsterone were dissolved in 0·15, 0·22 and 0·26 ml. of alcohol, and to each were added 0·2 ml. of 2 % *m*-dinitrobenzene solution and 0·19 ml. of 2*N* alc. KOH. Controls were made up to the same total volumes, viz. 0·54, 0·61 and 0·65 ml. The values of E_g after dilution at the end of 1 hr. were respectively: 0·65, 0·62 and 0·57. The effect of using 95 % alcohol instead of absolute alcohol as a diluent after incubation was to reduce the value of E_g for 0·1 mg. of androsterone from 0·365 to 0·35. With the earlier technique the difference was greater. The specifications in the routine method of amount of alcohol, and of content of aldehyde and water, were adopted as giving a sensitive reaction whilst setting limits attainable in practice.

Purity and concentration of m-*dinitrobenzene.* Ordinary *m*-dinitrobenzene gives a red colour with potassium hydroxide. This has been attributed to the presence of dinitrothiophene [Meyer & Stadler, 1884] which is detectable in a quantity of 0·1 μg. [Stadler, 1885] by this reaction. Nitration of "thiophene-free" benzene gave a product which still gave a colour with alkali, and we therefore followed the method of Willgerodt [1892] for removal of chromogenic material, details of which are given above. This method must be adhered to

strictly, for overheating, or prolongation of the alkali treatment, gives poorly crystallized products of low M.P. which are useless for colorimetry. One such sample, after repeated crystallization from alcohol, then ethyl acetate, yielded yellow crystals, sparingly soluble in alcohol, M.P. 148° (soft at 145°), identified as 3:3′-dinitroazoxybenzene (lit. M.P. 143°, 146·5°). (Found (micro-analysis by Dr G. Weiler): C, 50·36; H, 3·08; N, 19·9. Calc. for $C_{12}H_8N_4O_5$: C, 50·0; H, 2·8; N, 19·45 %.)

The effect of impurity in the *m*-dinitrobenzene on the value of E_g is shown by the figures in Table IV, dealing with the variation of E_g with amount of KOH when different samples of *m*-dinitrobenzene were used, the "purified" samples having passed through the alkali process.

The effect of the amount of *m*-dinitrobenzene used was investigated at two stages of the development of the final method. We first confirmed Wu & Chou's

Table IV. *Variation of* E_g *with amount of KOH*

Wt. of androsterone (mg.)	0·25	0·25	0·10	0·10	0·05
m-Dinitrobenzene ...	Unpurified	Purified	Unpurified	Purified	Purified
Vol. of 2*N* alc. KOH (ml.)	E_g				
0·09	0·32	—	—	—	—
0·12	0·47	0·365	—	—	0·12
0·15	0·54	—	—	0·28	—
0·16	—	0·53	—	—	—
0·19	0·56	0·63	—	—	0·165
0·20	—	—	0·32	0·34	—
0·25	0·51	0·66	0·34	0·365	0·20
0·275	—	—	0·345	—	—
0·30	—	0·61	—	0·34	0·20

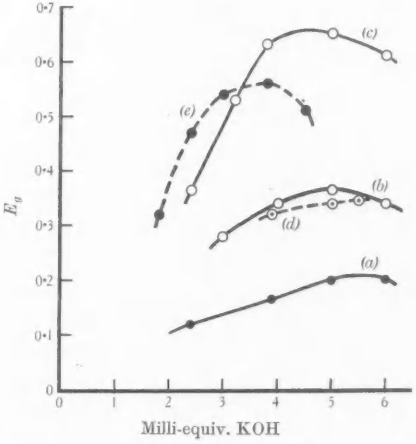

Fig. 3. Variation of E_g with amount of KOH. Curves (*a*), (*b*), (*c*): purified *m*-$C_6H_4(NO_2)_2$; 0·05, 0·1 and 0·25 mg. androsterone. Curves (*d*), (*e*): unpurified *m*-$C_6H_4(NO_2)_2$; 0·1 and 0·25 mg. androsterone.

observations that low values were obtained when small amounts of *m*-dinitrobenzene were used. In the final form of the reaction, 0·1 mg. of androsterone in 0·1 ml. of alcohol with 0·2 ml. of 2·5*N* KOH and varying amounts of 2 %

m-dinitrobenzene, viz. 0·14, 0·18, 0·20, 0·22 and 0·24 ml., made up to a total volume of 0·6 ml. with alcohol, gave the following values of E_g: 0·365, 0·36, 0·365, 0·375, 0·36. There was thus no systematic variation outside the limits of experimental error over this range of concentrations.

Effect of amount of potassium hydroxide. Series of tests were carried out in which androsterone was dissolved in $(0{\cdot}41-x)$ ml. of alcohol, 0·2 ml. of 2% alcoholic *m*-dinitrobenzene added, and then x ml. of $2N$ alcoholic KOH (x being varied from 0·09 to 0·3 ml.). Table IV gives the values of E_g for three series, one with an unpurified sample of *m*-dinitrobenzene, and these are shown graphically in Fig. 3.

The figure demonstrates particularly clearly the effect of purification of the *m*-dinitrobenzene. The proportion of KOH at which the absorption is maximal varies not only with the quality of the *m*-dinitrobenzene, but also with the amount of androsterone. The choice of 5 milli-equiv. of KOH in the standard method seems a satisfactory compromise, but the amount is evidently critical.

Effect of temperature and time of development. In general agreement with Wu & Chou, it was found that the absorption of the mixture diluted after 1 hr. increased by about 3% with a rise of 1° in the temperature of development.

With the technique modelled on that of Wu & Chou, it was found that with variation of the time of development the value of E_g rose to a maximum at about 1 hr., as shown by the series of figures in Table V, and there is no appreciable difference between 60 and 65 min.

Table V. *Variation of* E_g *with time of development of reaction mixture*

Time of development (min.)	0·05 mg. androsterone	0·10 mg. androsterone (*a*)	0·10 mg. androsterone (*b*)	0·10 mg. androsterone (*c*)	0·25 mg. androsterone (early technique)
30	0·17	0·315	—	—	0·45
45	0·20	0·35	—	—	0·51
50	—	—	0·355	0·35	—
60	0·20	0·39	0·375	0·37	0·61
65	—	—	0·375	—	—
70	—	—	0·37	0·36	—
75	0·19	0·39	—	—	0·60
90	—	0·365	—	—	—
105	0·18	0·34	—	—	—
120	0·16	0·35	—	—	—

Effect of light. When the reaction mixture was developed in bright sunlight the value of E_g for 0·25 mg. of androsterone was 0·31 as compared with $E_g=0{\cdot}61$ with development in dull, diffused daylight. On the other hand, further diminution of the illumination had no effect; the value $E_g=0{\cdot}60$ was obtained after development in the dark. (Early technique.)

Stability of colour after dilution. The colour fades slowly after dilution of the reaction mixture, and the fading appears to be accelerated by exposure of the solution to light or air. In diffuse daylight and with closed absorptiometer cells the values of E_g for 0·1 mg. of androsterone were 0·37 immediately after dilution, unchanged after 25 min., and 0·325 after 4 hr.

Similarly with 0·25 mg. of androsterone, when the solutions were poured back into test tubes and replaced in the thermostat in between measurements, the values of E_g immediately after dilution, and after 30 and 60 min., were 0·61, 0·57 and 0·52 respectively.

Remaining sources of error. A potential source of variation is water in the alcohol or in the potassium hydroxide. This factor has not been investigated.

The hidden sources of error responsible for the residue of uncontrolled variation probably include this, the quality of the *m*-dinitrobenzene and manipulative errors. The presence of small amounts of cholesterol has no effect. The presence of digitonin reduces the colour: 0·1 mg. of dehydroandrosterone and 0·5 mg. of digitonin gave a value of E_g corresponding to 0·08 mg. No errors due to alteration of the filters or of the photoelectric cells have been detected in control measurements of the absorption of an "orange" glass filter made over a period of a year.

(3) *Specificity—the reaction with other compounds*

Spectral colorimetry. Zimmermann [1936], using a Pulfrich photometer, plotted curves which were a rough indication of the absorption spectra of the reaction mixtures of androsterone, oestrone, testosterone and creatinine, using

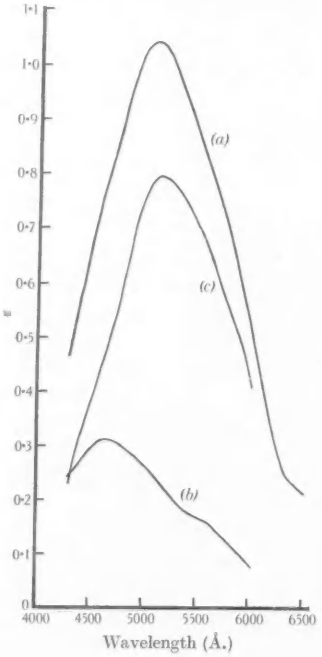

Fig. 4.

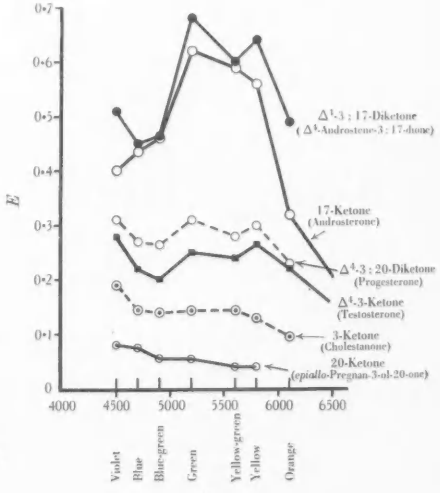

Fig. 5.

Fig. 4. Absorption spectra of (*a*) reaction mixture, 0·1 mg. of androsterone + *m*-$C_6H_4(NO_2)_2$ + KOH, and (*b*) of reagents alone, each against EtOH, with (*c*) calculated curve for test solution − blank. Hilger-Nutting spectrophotometer; 2 cm. cells; photometry of photographs at intervals of (*a*) $\epsilon = 0{\cdot}05$, and (*b*) $\epsilon = 0{\cdot}02$.

Fig. 5. "Absorptiometric spectra" with absorptiometer and Ilford "spectrum" filters of the reactions of typical steroid ketones + *m*-$C_6H_4(NO_2)_2$ + KOH.

measurements of the absorption within a limited range of wavelengths by inserting Zeiss "S" filters. We have used a similar method on a number of substances with the Spekker absorptiometer and Ilford "spectrum" filters. The

maxima of transmission of these filters, as given by the makers, occur at the following wave-lengths: violet, 4300 Å.; blue, 4700 Å.; blue-green, 4900 Å.; green, 5200 Å.; yellow-green, 5500 Å.; orange, 6000 Å. The range of transmission is of the order of 500–600 Å., except with the violet and orange filters, which are less selective. Measurements through these filters are designated below by the symbols E_v, E_b etc.

In the case of the reaction with androsterone, an accurate photographic measurement of the absorption spectrum has been made with a Hilger-Nutting spectrophotometer. Fig. 4 shows the absorption spectra of the reaction mixture obtained under standard conditions, in specially purified alcohol, with 0·1 mg. of androsterone (curve *a*) and of the blank, with reagents alone (curve *b*), comparison being made in each case with alcohol. The androsterone reaction shows a broad absorption band extending over the centre of the visible region, with a maximum at 5010 Å., whilst the reagents alone give low general absorption with a maximum at 4650 Å. Curve (*c*) shows the calculated difference between test and blank solutions. Reference to Fig. 5 shows that absorptiometric measurements with selective filters actually give a fairly faithful version of the net absorption spectrum after subtraction of the blank solution. The Ilford "spectrum-green" filter was selected for measurement of the absorption on the empirical ground that it gave the highest readings. The net absorption spectrum shows that any selective filter having maximum transmission somewhere between 5000 and 5400 Å. would be suitable for the purpose.

The reaction has been carried out generally under standard conditions, but in some cases using an earlier technique, with a number of methyleneketones, and the "absorptiometric spectra" have been measured with the Spekker absorptiometer and Ilford "spectrum" filters with the object of seeing whether compounds of different classes could be distinguished from one another. We are indebted to Messrs Ciba Ltd. for most of the androstane derivatives we have examined. The data obtained are in Table VI.

Influence of the position of the keto-group on the intensity and spectral characteristics of the colour. The first step in investigating the degree of specificity of the reaction with *m*-dinitrobenzene and alkali was to find whether it varied with the position of the keto-group in steroid compounds. Zimmermann [1936] had already shown that the approximate absorption spectrum given by testosterone was of a type different from that given by androsterone or oestrone, and Kaziro & Shimada [1937], using the Zimmermann technique, reported that the colour reaction was "positive" with 3-ketocholanic acid and "negative" with 6-, 7- and 12-ketocholanic acids.

The data we have obtained on various 3-, 6-, 17- and 20-ketones, given in detail in Table VI, show that the production of an intense, broad absorption band with a maximum in the green, is, among the steroid compounds, characteristic of the 17-ketones. In none of the groups of compounds is the type or intensity of the absorption much affected by distant substituents, and the spectra plotted in Fig. 5 may be taken as representative of their classes.

Among the *17-ketones*, we have investigated dehydroandrosterone in some detail with respect to factors influencing the intensity of the reaction on the same lines as androsterone. The effects of altering the concentration of the reagents and the time of development were the same as with androsterone. Dehydroandrosterone and oestrone gave calibration curves which followed closely that of androsterone. Androstan-17-one and $\Delta^{3,5}$-androstadien-17-one were very kindly given to us by Dr F. L. Warren of the Royal Cancer Hospital, and we take this opportunity of thanking him.

Table VI. *"Absorptiometric spectra" of steroid and other methyleneketones and of neutral fractions of urine extracts with* m*-dinitrobenzene and KOH*

Class and substance	Wt. (mg.)	Time of development	Absorptiometer readings (test – blank) and wavelength examined: E_v (max. 4500 Å.)	E_b (max. 4700 Å.)	E_{b-g} (max. 4900 Å.)	E_g (max. 5200 Å.)	E_{y-g} (max. 5600 Å.)	E_y (max. 5800 Å.)	E_0 (max. 6100 Å.)
17-Ketones:									
Androsterone	0·1	1 hr.	0·21	0·26	0·305	0·385	0·35	0·31	0·18
	0·2	1 hr.	0·40	0·435	0·46	0·62	0·59	0·56	0·32
Dehydroandrosterone	0·2	1 hr.	0·30	0·34	0·42	0·58	0·475	0·44	0·265
Androstan-17-one	0·1	1 hr.	0·19	0·23	—	0·37	—	—	—
	0·2	1 hr.	0·365	0·41	0·47	0·62	0·555	0·53	0·135
$\Delta^{3:5}$-Androstadien-17-one	0·1	1 hr.	0·20	0·245	—	0·38	—	—	—
	0·2	1 hr.	0·43	0·45	0·48	0·67	0·61	0·61	0·355
Oestrone	0·1	1 hr.	—	—	—	0·42	—	—	—
	0·2	1 hr.	0·39	0·45	0·46	0·66	0·60	0·585	0·345
3-Ketones:									
Androstan-17-ol-3-one	0·2	20 min.	0·26	0·245	0·265	0·31	0·33	0·32	0·23
	0·2	1 hr.	0·225	0·18	0·17	0·21	0·19	0·19	0·135
Cholestanone	0·2	2 min.	0·18	0·195	0·205	0·26	0·285	0·265	0·175
	0·2	5 min.	0·26	0·23	0·24	0·295	0·33	0·325	—
	0·2	10 min.	0·235	0·21	0·22	0·28	0·31	0·31	0·22
	0·2	20 min.	0·20	0·175	0·175	0·23	0·25	0·265	0·185
	0·2	40 min.	0·16	0·115	—	0·15	0·145	0·15	—
	0·2	1 hr.	0·19	0·145	0·14	0·145	0·145	0·13	0·095
Δ^4-3-Ketones:									
Testosterone	0·1	1 hr.	0·18	0·15	—	0·155	0·15	0·165	—
	0·2	1 hr.	0·28	0·22	0·20	0·25	0·24	0·265	0·22
Cholestenone	0·1	10 min.	0·04	0·02	—	0·03	—	—	—
	0·1	20 min.	0·08	0·05	—	0·06	—	—	—
	0·1	30 min.	0·085	0·08	—	0·075	—	—	—
	0·1	1 hr.	—	—	—	0·135	—	—	—
	0·2	1 hr.	0·27	0·22	0·20	0·24	0·23	0·245	0·20

3:17-Diketone:									
Androstane-3:17-dione	0·1	1 hr.	—	—	0·33	0·41	0·37	—	—
	0·2	1 hr.	0·53	0·47	0·52	0·68	0·62	0·62	0·41
Δ^4-3:17-Diketone:									
Δ^4-Androstene-3:17-dione	0·1	1 hr.	—	—	—	0·42	—	—	—
	0·2	1 hr.	0·51	0·45	0·465	0·68	0·60	0·64	0·49
20-Ketones:									
epiallo-Pregnan-3-ol-20-one	0·1	1 hr.	—	—	—	0·045	—	—	—
	0·2	1 hr.	0·085	0·075	0·055	0·055	—	—	—
3:20-Diketone:									
Progesterone	0·1	1 hr.	0·20	0·19	—	0·19	—	—	—
	0·2	1 hr.	0·31	0·27	0·265	0·31	0·28	0·30	0·23
6-Ketones:									
Cholestan-6-one	0·2	1 hr.	—	—	—	0·01	—	—	—
Cholestane-3:6-dione	0·2	1 hr.	0·14	0·105	·09	0·11	0·10	0·115	0·09
Other compounds:									
*cyclo*Pentanone	0·058	5 min.	0·35	0·445	0·53	0·70	0·62	0·62	0·46
	0·058	10 min.	0·32	0·35	0·405	0·57	0·53	0·49	0·35
	0·058	20 min.	0·335	0·34	0·35	0·47	0·43	0·395	0·30
	0·058	30 min.	0·33	0·29	0·31	0·44	0·40	0·385	0·29
	0·058	1 hr.	0·38	0·32	0·30	0·40	0·36	0·33	0·25
*cyclo*Hexanone	0·067	1 hr.	0·35	0·22	0·175	0·19	0·14	0·12	0·18
Acetophenone	0·08	1 hr.	0·42	0·46	0·52	0·66	0·50	0·33	0·15
Acetone	0·04	1 hr.	0·295	0·29	0·27	0·29	0·22	0·20	0·15
Urine extracts (bulk collections)									
Female FU. 57.B	0·02 l.	1 hr.	0·18	0·19	0·205	0·27	0·24	0·21	0·135
FU. 62.B	0·01 l.	1 hr.	0·21	0·21	0·245	0·29	0·28	0·24	0·15
FU. 76.B	0·01 l.	1 hr.	0·30	0·30	0·33	0·43	0·405	0·36	0·225
Male MU. 38.B	0·01 l.	1 hr.	0·26	0·26	0·29	0·38	0·335	0·295	—
MU. 39.B	0·01 l.	1 hr.	0·23	0·225	0·265	0·32	0·30	0·26	0·165
MU. 75.B	0·01 l.	1 hr.	0·225	0·21	0·265	0·37	0·30	0·26	0·15

Saturated 3-ketones show a characteristic rapidity in development of the colour, which then fades, and after 1 hr. of development only a very low, general absorption is shown. The behaviour of cholestanone was investigated in detail, and the most intense colour, characterized by a band in the yellow and yellow-green, is given after only 5 min. development. This behaviour seems to be common to compounds having the group —CH_2—CO—CH_2—. *cyclo*Pentanone, for a specimen of which we are indebted to Prof. R. P. Linstead, gives a band with maximum in the green, which is developed rapidly.

Δ^4-*3-ketones*, in contrast to the saturated 3-ketones, require a long time of development, and, in the case of cholestenone, some detailed figures for which are given in Table VI, it seems that development of the maximum colour is not complete after 1 hr. All the Δ^4-3-ketones show a characteristic peak in the yellow, in addition to one in the green, and this is visible even when the powerfully chromogenic 17-keto-group is also present, as in Δ^4-androstene-3:17-dione (cf. Fig. 5).

The *20-keto-group* gives a very low, but measurable general absorption. In combination with a Δ^4-3-keto-group it causes a small general rise in the absorption.

The *non-steroid* compounds were measured in amounts which were the molar equivalents of 0·2 mg. of androsterone. Acetone shows the rapid development of colour characteristic of dimethyleneketones. Acetophenone is noteworthy for the resemblance of the curve to that of 17-ketosteroids, except for considerably lower absorption in the yellow and yellow-green. This resemblance, in spite of a considerable difference in structure, demonstrates with what caution absorption spectra of this colour reaction should be accepted as indicative of structure without confirmatory evidence.

Absorption spectra of the colour reaction with urine extracts. The absorptiometer measurements obtained with extracts from bulk collections of male and female urines are given in Table VI and plotted in Fig. 6 where they are compared with the absorptiometric spectrum of 0·1 mg. of androsterone. It is evident that there is no characteristic difference between extracts from either male or female urine and a typical 17-ketosteroid beyond the minor one of having a value for E_v which is relatively higher and of the same order as E_b. However, in certain abnormal cases there are major differences in the absorption in the shorter wavelengths. We have, in practice, made measurements of E_v and E_b as a routine, in addition to E_g. Certain urines yield extracts which give at $E_g = 0{\cdot}37$ values of E_v or E_b or both which are of the same order as E_g ($E_v/E_g > 0{\cdot}8$). It is clear that these contain chromogenic compounds not present in appreciable amount in normal urine, and, since the value of E_g is liable to be affected by absorption in the adjacent regions of the spectrum, there must be some doubt whether such extracts can fairly be compared with others on a scale of values of E_g. The ratio E_v/E_g tends to be high when the chromogen content is low, and is probably then an indication of the presence of a substance giving rise to general absorption. There is also qualitative evidence, from the rate of development of colour with urine extracts, that substances like saturated 3-ketosteroids are present in small amount. Although in certain cases an overestimate of the biological activity was made when the value of E_v/E_g was high, in other cases, in which, presumably, the urine component giving absorption in the violet did not affect E_g, the relation was normal, and no general rule can be laid down except to regard with suspicion an extract with a high value of E_v/E_g. In the following treatment of the correlation of colorimetry and biological assay no selection of urine extracts in respect of values of E_v/E_g has been made. In two

cases the abnormal colour was traced to contamination resulting from extraction of vulcanite stoppers by toluene used to preserve the urine. Naturally, such contamination must be avoided in collecting specimens intended for colorimetric assay.

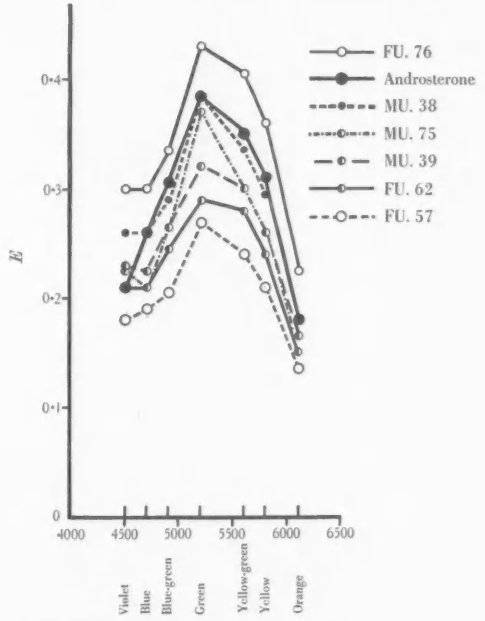

Fig. 6. "Absorptiometric spectra" of the reaction of extracts of men's (MU.) and women's (FU.) urine + m-$C_6H_4(NO_2)_2$ + KOH compared with 0·1 mg. of androsterone. (FU. 57 equiv. 0·02 l.; other urine extracts equiv. 0·01 l.)

(4) *Correlation of colorimetry and biological assay*

Method of biological assay. The urine extracts in oily solution have been assayed for androgenic activity by a capon comb-growth method, described in detail elsewhere [Emmens, 1938]. A dose/response curve was constructed by giving injections of international standard androsterone, and checked regularly by at least one control group of 5 capons. Androgenic activity is expressed as being equivalent to so many international units of androsterone per litre (1 I.U. = 100 μg.), and the estimates based on groups of five birds. In four instances an extract has been assayed by the direct inunction of an oily solution on the comb, and compared with androsterone similarly administered. These four estimates fall well within the range of the others, there being therefore no reason to exclude them from the calculations, despite the difference of technique. Their influence on any conclusions drawn from the data is in any case negligible.

Urines investigated. In the course of the scheme of research of which this investigation forms a part, a variety of urines has been extracted and examined, including bulk collections from groups of normal men or women, a small number from men and women in hospital with no obvious sexual dysfunction, and urine

from eunuchs, eunuchoids, ovariectomized women, cases of Addison's disease and hirsutism in females. For the purpose of considering whether colorimetric and biological assays could be correlated, all these have been lumped together; only cases in which hormone-producing tumours have been proved to be present have been excluded. It may therefore be considered that the hypothesis of correlatability is put to a severe test. The possibility of distinguishing between these classes of patients will not be discussed here.

Statistical treatment of the results

Fig. 7 is a scatter-diagram showing the correlation between the colorimetric and biological assay figures for 59 urine extracts, which are given in Table VII. These were obtained from collections from the following: normal men, 4 groups

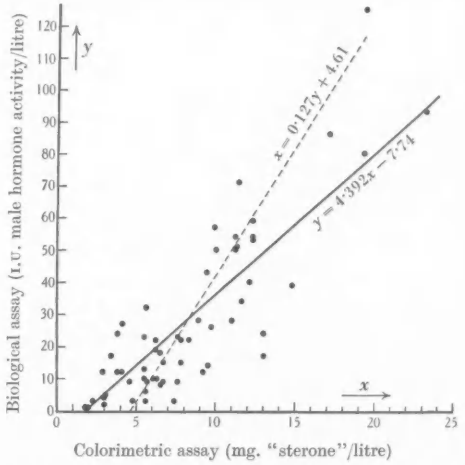

Fig. 7. Correlation of capon comb-growth assay and colorimetric assay of 59 urine extracts.

and 7 individuals; normal women, 4 groups and 6 individuals; eunuchs, 8 (9 samples); ovariectomized women, 8; eunuchoids, 2; hirsute women, 8; cases of Addison's disease, 7 (6 samples from 4 women; 3 men); carcinoma of cervix, 2. To provide comparable figures for calculation, all quantities have been expressed as per litre, though the quantities per day are the significant ones for individual patients and have been measured in most cases. A correlation coefficient has been calculated by grouping the data with units equal to 10 I.U. and 2 mg. "sterone", this coefficient being 0·745. The two regression lines, $y=4{\cdot}392x-7{\cdot}74$ and $x=0{\cdot}127y+4{\cdot}61$ express, respectively, the regression of content of international units by capon assay (y) on "sterone" content (x), and that of "sterone" content on international unit content. The appearance of a better "fit" given by the first of these lines, and the choice of it as a basis for discussion of the meaning of the results depends on the relative errors of the colorimetric and biological tests.

It is known that the error of the capon comb-growth test may be large, but a satisfactory estimate of the average value of this error for a large number of tests is almost impossible to obtain since it depends on both the age of the

Table VII. *Colorimetric and biological assays of urine extracts*

Type and ref. No.	mg. "sterone"/l.	I.U./l.	Type and ref. No.	mg. "sterone"/l.	I.U./l.
(*i*) 1	1·8	1	(*f*) 16	7·6	9
(*j*) 11	2·0	1*	(*d*) 21	7·6	23
(*i*) 3	2·3	3	(*b*) 84	7·8	15
(*j*) 6	2·9	12	(*e*) 72	7·8	22
(*e*) 64	3·0	2*	(*c*) FU. 62	8·3	22
(*e*) 77	3·0	4	(*a*) MU. 75	8·9	28
(*f*) 14	3·1	5	(*f*) 15	9·2	12
(*j*) 8	3·4	17	(*b*) 31	9·4	43
(*c*) FU. 57	3·8	12	(*h*) 25	9·5	14
(*d*) 51	3·8	24	(*d*) 31	9·7	26
(*b*) 61	4·1	27	(*a*) 44	9·9	57
(*e*) 68	4·2	12	(*f*) 10	10·0	50
(*i*) 7	4·6	9	(*c*) FU. 76	11·0	28
(*h*) 21	4·8	3	(*k*) 50	11·2	50
(*h*) 27	5·5	10*	(*a*) 65	11·2	54
(*f*) 9	5·5	13	(*b*) 2	11·3	51
(*i*) 9	5·5	23	(*d*) 11	11·4	71
(*e*) 98	5·6	3	(*a*) MU. 38	11·6	34
(*e*) 86	5·6	6	(*h*) 26	12·1	40
(*b*) 11	5·6	32	(*b*) 21	12·3	53
(*e*) 104	5·7	9	(*g*) 40	12·3	54
(*d*) 81	6·1	10	(*g*) 33	12·3	59
(*f*) 12	6·2	19	(*h*) 31	13·0	17
(*i*) 4	6·2	22	(*k*) 51	13·0	24
(*c*) 38	6·3	10	(*d*) 121	14·8	39
(*f*) 11	6·5	18	(*h*) 38	17·1	86
(*e*) 65	6·55	8	(*h*) 34	19·3	80*
(*i*) 5	6·7	9	(*f*) 13	19·4	125
(*b*) 81	6·7	15	(*h*) 24	23·2	93
(*e*) 73	7·4	3			

(*a*) Bulk collection, normal men.
(*b*) Normal men.
(*c*) Bulk collection, normal women.
(*d*) Normal women.
(*e*) Eunuchs.
(*f*) Ovariectomized women.
(*g*) Eunuchoids.
(*h*) Hirsute women.
(*i*) Addison's disease, women.
(*j*) Addison's disease, men.
(*k*) Carcinoma of cervix.

* Capon assay by inunction.

birds and the amount of growth stimulated [Emmens, 1938]. However, 20 control groups receiving androsterone for a period during which capon variability was on the high side, and during which approximately one half of the estimates used here were made, yield a correlation of 0·862 between comb-growth and the amount of androsterone injected. The correlation for the whole period is certainly not less than this, and probably as high as 0·9. The z test [Fisher, 1932] reveals that a correlation of 0·745 derived from 59 pairs (the number of extracts assayed) is not significantly different from one of 0·862 derived from 20 pairs. One can conclude therefore that the correlation between biological and colorimetric assays is not much less than that between biological assay and the true amount of androgenic material present, even when the latter is pure androsterone.

The correlation between values of E_g and the amount of androsterone present cannot be accurately expressed by a correlation coefficient, since the relationship is not linear. Fig. 1 demonstrates that the colorimetric determination of androsterone is very accurate, although the accuracy with which urine extracts have been assayed is appreciably less than that indicated in Fig. 1 and the calculation on p. 1315 suggests. There remains no doubt, however, that the greater part of the departure of the correlation between colorimetric and biological assays from unity is due to the errors of biological assay. The small contribution of the errors of colorimetric assay to this departure probably does

not account completely for the remainder, since a perfect fit of the biological and colorimetric estimates could be expected only on uniform material. Thus, if it were assumed that androsterone and dehydroandrosterone were the active substances, differences from sample to sample in the ratio of these two compounds would reduce the correlation of the two estimates, unless balanced by appropriate variations in the content of biologically inactive chromogens. In view of the fact that a given colorimetric reading may be obtained from a series of mixtures of different active and inactive chromogens, each giving a different result when tested biologically, the degree of correlation found is high.

Chemical evidence

Certain preliminary work has been carried out on the analysis of urine extracts into their constituents with a view to the complete identification of the latter; results have been obtained which, subject to the reservations necessitated by the inherent error of capon assay, and the rather unquantitative character of the methods, indicate the association of biological activity with the chromogenic, ketonic fraction. Separation of the ketonic fraction as water-soluble compounds by treatment with Girard's reagent T (trimethylammonium acethydrazide chloride) [Girard & Sandulesco, 1936] gives results such as the following:

450 mg. of extract MU. 75[1] equivalent to 10·25 l. of urine, assayed at 8·9 mg. of "sterone" per litre by colorimetry, or 28 international units of androgenic activity per litre by capon comb growth, yielded, after extraction with methanol and two treatments with Girard's reagent, (*a*) a high-ketone fraction, wt. 86 mg., "sterone" content 6·8 mg. per litre, androgen activity 45 I.U. per litre, (*b*) two low-ketone fractions, one, weight 12 mg., "sterone" 0·19 mg. per litre, and the other, weight 225 mg., with a "sterone" content of 0·37 mg. per litre (but with a high value of E_v), and androgen activity less than 2 I.U. per litre. This shows clearly that the androgenic compounds are confined to the ketonic fraction.

Discussion

The use of "male hormone assay" of urine extracts by biological methods as a method of diagnosis of the physiological condition of a patient entails the assumption, originally based, perhaps, on a presumed specificity of male hormone activity, that the circulating hormones, although they may be destroyed or transformed in the course of excretion, will yet give a product whose activity will be proportional to the physiological activity of the original compounds present in the human body. An incidental assumption is made in supposing that the usual (and only practical) method of assessing the biological activity by the comb-growth of capons gives a measure of the activity in the human body. The factual support for the main assumption is slender, owing to the immense difficulties of chemical analysis of extracts of tissue, blood and excreta. The only compounds with marked androgenic activity so far isolated from the organism are testosterone from the testis, and Δ^4-androstene-3:11:17-trione from adrenal cortical tissue. The only active compounds which have been isolated from normal urine are androsterone and *trans*dehydroandrosterone, or Δ^4-androsten-3-*trans*-ol-17-one, and the amounts identified account for only a fraction of the biological activity. Urines from adrenal tumour cases have

[1] We are indebted to Messrs Boots Pure Drug Co. for this extract made at Nottingham from a 250 l. bulk collection of men's urine. Other data from this extract have been used in Tables VI and VII.

yielded $\Delta^{5:7}$-androstadien-17-one [Burrows *et al.* 1937], and *trans*dehydroandrosterone [Callow, 1936, 2]; in the last case the amount of the compound isolated accounted for 70% of the biological activity. A related, inactive compound, *epi*aetiocholanediol, has been isolated in very small amount from normal urine extract [Butenandt *et al.* 1937]. The genetic relationship between secretion and excretion is thus far from clear with respect to chemical structure. The investigation of the relationship with respect to biological activity is as yet hardly begun, though from the work of Kochakian [1937] and from work carried out in this laboratory in collaboration with clinicians, a preliminary account of which has been given in a lecture by one of us [Callow, 1938], it is already evident that the recovery of androgenic activity from urine extracts is only of the order of 5%, as a maximum, of that administered by injection. Here again, the scale of comparison is comb growth in the capon, although it is known that the relative activities of androgens, which vary even in capons according to the mode of administration, are different in laboratory mammals, and, presumably, in man. Further, the biological assay is of a mixture of substances, and, of two known constituents of this mixture, one has about three times the activity of the other.

It seems, therefore, that an examination of urine extracts by a chemical test, to which the known active urine constituents and closely related steroids respond, will entail no more dangerous assumptions than examination by biological assay.

The first result of this work is that of increasing the accuracy and reproducibility of a method of determining 17-ketosteroids. It has then been shown that a greater degree of specificity can be introduced into this determination by rough measurements of the absorption spectra. A claim for complete specificity would be untenable unless a more exact examination of a far wider range of compounds had been made. The hypothesis that the method gives a measure of the 17-ketosteroids in urine extracts is justified and supported by three considerations. First, the method of obtaining the extracts is such that neutral, non-volatile compounds of high molecular weight are separated. Secondly, after chemical fractionation of the extract, the chromogenic activity is concentrated in the ketonic fraction. Thirdly, the absorption spectra of the coloured compounds formed by this material with *m*-dinitrobenzene in alkali closely resemble those of the compounds formed by pure 17-ketosteroids.

The further hypothesis that a determination of 17-ketosteroids made in this way gives a measure of the androgenic activity is suggested in the first place by the knowledge that two of the compounds to which urine extracts owe their androgenic activity are actually 17-ketosteroids. Secondly, there is the inherent likelihood that 17-ketosteroids will all possess androgenic activity. Confirmation is afforded by the degree of correlation between colorimetric and capon assays. When it is considered that the urine extracts are certainly a complex mixture of compounds with both androgenic and chromogenic properties, and that the first step in obtaining them is a vigorous acid hydrolysis involving both liberation and destruction, which may be selective, under conditions difficult to control, and, further, that an absorption spectrum is a physical property particularly sensitive to impurities, it might well be expected that even if the natural urine contained a simple mixture of androgens in constant proportions, the inherent correlation between colorimetric and biological assays would be blurred beyond recognition. It has been shown that a significant degree of correlation does exist. Thus, if it is a question of deciding, on theoretical grounds of chemical metabolism or physiological relationship, whether chemical or biological assay is the better, we can make the best of both worlds, and employ either. Neither

form of assay is specific, but the chemical one is more susceptible to investigation and improvement in this respect, and has the added advantage of rapidity and smaller inherent error: it would seem at least as likely to give diagnostically significant results.

The contributions of this investigation to the question of the exact composition of the urinary excretory transformation products of male hormones are an improved chemical test for a type of these products, but not for any individual compound, and an indication of a relationship of chromogenic to biological activity concordant with a simple working hypothesis without giving it preferential support. The relationship $y = 4{\cdot}392x - 7{\cdot}74$, which gives the most likely estimate of the biological assay equivalent to a given colorimetric reading, is intermediate between that which would be given by androsterone and dehydroandrosterone. In practice pure androsterone would give an equation close to $y = 10x$, and pure dehydroandrosterone one approximating to $y = 3{\cdot}3x$, since the activity of the latter on capons is about one-third of that of androsterone. It might be assumed that the androgenic and chromogenic material in urine extracts is a mixture of androsterone and dehydroandrosterone, the two substances which have actually been isolated from normal male urine, an assumption which has been made, on other grounds, by Dingemanse & Laqueur [1938]. However, the intermediate position of the experimentally determined line is not in itself proof of the presence of a mixture of androsterone and dehydroandrosterone in the average urine dealt with. It could result from the presence of androsterone and biologically inactive chromogen only. The presence of some inactive chromogen is indicated by the constant 7·74, which is a measure of the amount found when little active substance is present, whilst there may be more inactive chromogen in urines with higher biological activity. Moreover, there is a wide discrepancy between the amounts of androsterone and dehydroandrosterone recorded as actually isolated (about 1 mg. per litre of each) and the amounts indicated by biological assay. From the evidence now presented, no more definite conclusion can be drawn, than that the content of biologically active material is proportional to the content of 17-ketosteroid compounds.

Summary

1. A description is given of a modified and improved method of determining androsterone and other 17-ketosteroids by the colour reaction with *m*-dinitrobenzene and potassium hydroxide in alcoholic solution.

2. The sources of error and the factors influencing this determination have been investigated.

3. Examination of the behaviour of other methyleneketones has shown that the absorption spectrum of the coloured solution produced and the rapidity of development of the colour vary according to the structure of the compound.

4. The neutral fractions of extracts of hydrolysed urine give the colour reaction characteristic of 17-ketosteroids, although with some indication of the presence of other chromogenic compounds. Further, biological activity is associated with the ketonic fraction. These facts justify the use of the reaction for estimating the excretory transformation products of male hormones.

5. A comparison of the chemical with the biological method of determining the excretory products by their activity in the capon comb-growth test has been made with 59 urines from a variety of sources. A significant degree of correlation is found between the colorimetric assay expressed in chromogenic equivalents

of androsterone ("sterone") per litre of urine and the biological assay expressed in international units of male hormone activity per litre.

6. It is concluded that colorimetric assay can replace biological assay provided that due regard is paid to the occasional presence of interfering substances, which may be revealed when the approximate absorption spectrum is examined.

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CLXXVII. THE EFFECTS OF PANCREATIC EXTRACTS ON FAT DEPOSITION IN THE DIETARY FATTY LIVER

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Allan *et al.* [1924] reported that depancreatized dogs receiving adequate amounts of insulin and maintained on a diet of lean meat, sucrose and bone ash did not survive for periods of more than a few months. They also observed that the symptoms of failure of liver function due to fat infiltration of the liver found in such animals, could be prevented by adding raw pancreas to the diet. They suggested, therefore, that the pancreas might possibly produce an internal secretion necessary for the physiological integrity of the liver. The subsequent discoveries of Hershey [1930] that lecithin could successfully replace raw pancreas in the diet of depancreatized dogs, and of Best & Huntsman [1932] that choline and betaine could prevent and cure fatty infiltration in the livers of rats fed on high fat diets have been extended in many directions. So far as we are aware, however, no proof has yet been advanced that the active agent of the pancreas is, in fact, choline, and particular interest therefore attaches to the claims of Dragstedt and his colleagues [Dragstedt *et al.* 1936, 1, 2] that raw pancreas contains an active substance other than choline and that this substance, to which they give the name "lipocaic" may be concentrated in dilute alcoholic extracts of the tissue.

The work described in this paper was undertaken in an attempt to extend these findings to the dietary fatty liver of the rat, which lends itself to more accurate experimentation than that of the depancreatized dog.

While these experiments were in progress, other workers reported the results of similar studies [Chaikoff & Kaplan, 1937; Kaplan & Chaikoff, 1937; MacKay, 1937; Shapiro & Wertheimer, 1937; Aylward & Holt, 1937; MacKay & Barnes, 1938; Best & Ridout, 1938]. Some of these results throw doubt on the existence of a pancreatic factor other than choline influencing fat deposition in the liver, while in some cases the conclusions drawn appear to us to be based on inadequate data. These points are discussed later in this paper.

The general plan of the experiments has been to feed control groups of rats on diets which cause fatty livers. At the same time other groups have received the same diet with the addition of varying amounts either of pancreatic extract or of choline, in order to assess in terms of choline the activity of pancreatic extract in preventing fat deposition. It is difficult to strike the correct range of choline dosages in attempting to assay an unknown substance and many more groups of animals were used than are recorded in the tables which follow. Tables II–V contain, therefore, only the results of the 42 groups which are relevant to the discussion, either because they provide the actual results on which the effect of the pancreatic extract has been assessed, or alternatively because it is important in forming a decision as to the activity of the pancreatic

extracts, that the effects of increasing amounts of choline should be recorded for comparison, in order that the accuracy of the assay may be suitably appraised.

Because of the differences of opinion evident from the publications referred to above, it appeared to us necessary to obtain a considerable body of evidence. Accordingly six experiments were carried out, three on the "cholesterol", and three on the "fat" fatty liver.

Experimental

Preparation of the extracts

Fresh ox pancreas obtained from the slaughter house was freed from extraneous connective tissue and fat.

Dragstedt *et al.* [1936, 2] extracted pancreas with saline and with alcohol of various dilutions and found that saline or 40 % alcohol gave more active extracts than 70 % alcohol. In the experiments now reported the method of extraction used has followed closely that of these workers except in two particulars: (*a*) in the initial extraction of each batch alcohol was added so that, allowing for the water content of the pancreas, the final concentration was 40 %, all the succeeding extractions being carried out with 40 % alcohol save in Exp. 2; (*b*) instead of evaporating the extracts in open dishes and extracting the residual solid with ether, alcohol and water were removed *in vacuo* and extraction carried out by shaking with ether in a funnel before the material became pasty. In all the experiments the extracts were made up to a definite volume, and stored in the refrigerator: aliquots providing sufficient of the extracts for incorporation in 3-day portions of diet for all groups receiving pancreatic extract were withdrawn into test tubes, pasteurized, sealed and stored in the refrigerator for use as required.

Exp. 1. 1·2 kg. of minced pancreas were left overnight with 2·4 l. of 60 % alcohol, strained through muslin and twice re-extracted with 40 % alcohol (2·4, 2·4 l.); the residue was treated with ether (2·5, 2·0, 2·0 l.). The first "ether" extract which contained much alcohol and water was added to the 40 % alcohol extracts and the whole evaporated *in vacuo* at 60°. Fat was removed from the concentrate by shaking with the second ethereal extract of the protein residue and four times subsequently with fresh ether (1 l.); alcohol and ether were then removed from the aqueous suspension *in vacuo* and the volume made up to 1·2 l. with water.

Exp. 2. 5 kg. minced pancreas were treated three times with 40 % alcohol as in Exp. 1: the protein residue was then extracted with 95 % alcohol (2, 3 l.), the extracts evaporated *in vacuo* and added to the previous extracts. Much of the fat thus added to the 40 % alcohol extracts was removed by ether, but the high choline content (Table I) indicates that some phosphatide was probably left in the aqueous layer.

Exp. 3. 1·2 kg. of minced tissue worked up as in Exp. 1.

Exp. 4. The extract prepared for Exp. 3 was used. In addition, the effect of precipitation with trichloroacetic acid was investigated. 40 ml. of the extract of Exp. 3, equivalent to 96 g. of pancreas, were treated with an equal volume of 10 % trichloroacetic acid, and the precipitate filtered off. The filtrate was extracted six times with ether to remove trichloroacetic acid, evaporated and then made up to 96 ml. The precipitate represented 6·4 % of the solids taken, and the filtrate 77·4 %.

Exp. 5. 250 g. of minced tissue extracted as in Exp. 1.

Exp. 6. 2 kg. of minced tissue extracted as in Exp. 1; evaporation of the extracts at a bath temperature of 30–40° was continued to dryness and the solid extracted with 500 ml. ether. The residual solid after removal of ether was dissolved in 500 ml. water.

The choline content of each extract was determined by biological assay on the isolated rabbit intestine after hydrolysis and acetylation. It was thus

possible to decide on the amount of choline to be given to the groups receiving choline, in order to assess the non-choline activity of the pancreatic extracts. In addition, the total solids present in each extract, save in that of Exp. 5, were obtained by drying to constant weight at 105°, and the ash content determined. In Exps. 1, 2 and 6 estimations of total N and in the former two of non-protein-N on the filtrate obtained after precipitation with sodium tungstate and H_2SO_4 were made. The results of these analyses are recorded in Table I.

Table I. *The solids of the pancreatic extracts*

Exp.	Yield % fresh pancreas	% of total solids			
		Choline	Total N	N.P.N.	Ash
1	5·5	1·0	10·9	8·0	13·5
2	12·7	2·45	11·8	10·8	8·7
3	10·45	1·64	—	—	10·1
4	10·45	1·64	—	—	10·1
Trichloroacetic acid filtrate	8·08	2·12	—	—	10·8
5	(1·8 mg. choline/g. pancreas)		—	—	—
6	4·64	3·3	9·5	—	16·0

Animal feeding

In Exps. 1, 2 and 6 diets which result in "cholesterol" fatty livers, and in Exps. 3, 4 and 5 diets producing "fat" fatty livers were employed. The diets and the periods of feeding were as follows.

Exp. 1. Caseinogen (alcohol-extracted) 5, cholesterol 2, beef dripping 29, cod liver oil 1, glucose 53, marmite 5 and salts 5 parts; 21 days.

Exp. 2. As in Exp. 1 for 15 days.

Exp. 3. Caseinogen 5, beef dripping 39, cod liver oil 1, glucose 50, salts 5 parts with 10 μg. aneurin per rat per day; 16 days.

Exp. 4. As in Exp. 3 save that the caseinogen was increased to 8 parts at the expense of 3 parts of glucose, while after 7 days it was replaced by commercial egg albumin; 16 days.

Exp. 5. As for the first 7 days of Exp. 4 save that the daily intake of aneurin was increased to 12 μg. and each rat also received 24 μg. lactoflavin; 15 days.

Exp. 6. Commercial egg albumin 8, beef dripping 39, cod liver oil 1, glucose 45, cholesterol 2, salts 5 parts; each rat received 25 μg. aneurin daily; 19 days.

The supplements of pancreatic extract and of choline as chloride were dissolved in the same volumes of water and incorporated in 3-day portions of the dry diets: for the control diets an equal volume of water was used.

At the beginning of the experiments, all the groups except two control groups contained 10 animals (5 ♂, 5 ♀), suitably chosen so that animals of the same weight and sex appeared in each group. At the conclusion of the feeding period, animals which showed a weight loss greater than 20% or any other abnormality were rejected. As a result, when the animals were killed 35 of the groups each contained either 9 or 10 animals, groups 14, 21 and 22 contained 8, and groups 28 and 38 only 7; two control groups 18 and 27, which began as groups each of 20 animals, were finally reduced to 18 and 19 respectively.

Analysis of livers

The methods of investigating the livers and the analysis of the lipoids were as follows. In Exps. 1, 2 and 6 in which the "cholesterol" fatty liver was employed, the total ethereal extract of the pooled livers of each group of animals was prepared. In two of these experiments, it was completely analysed for

phosphatide, cholesterol, cholesteryl esters and glyceride. Since only the glyceride fraction is pertinent to the discussion of the results, the amounts of the total lipoids and glyceride only are recorded (Tables II and V). In Exp. 2 a detailed analysis was not made, fatty acids and unsaponifiable matter only being determined after saponification. In this experiment the total fatty acids and the total lipoids only are recorded (Table III). Previous experience has shown that for all practical purposes differences in the total fatty acid contents of the livers of the different groups may be regarded as differences in glyceride. In Exps. 3–5 in which the "fat" fatty liver was investigated the livers of all the animals were individually analysed by the saponification method for their content of fatty acids and unsaponifiable matter. The "fat" contents of the different groups of these three experiments recorded in Table IV are thus the mean values of the fatty acids and unsaponifiable matter of the individual animals of each group.

Food intake

In Exp. 1 the daily food intake of all the animals was measured, and in Table II the amounts of pancreatic extract or of choline recorded are the actual amounts ingested by the different groups. In Exps. 2–6 the daily food consumption was not determined and the amounts of pancreatic extract or choline recorded in Tables III–V have been calculated on the basis that each rat consumed 8 g. of diet per day. Much previous experience suggests that this is a reasonable figure for purposes of calculation for animals of the weight used: further, since the average weight losses in the various groups of each experiment were similar, no serious error will be introduced in using such calculated values for comparative purposes, as will be shown later. In the tables the amount of pancreatic extract is recorded as the daily intake of the weight of pancreas from which it was derived ("pancreas equivalent"), and also as the daily intake of its contained solids ("actual weight"). The "extra choline intake" represents the choline additional to that present in the basal diet received by the different groups either in the pancreatic extract or as administered choline chloride.

Results

When a liver becomes fatty, the percentage of fat in it increases and at the same time its size increases. It is thus possible to discuss the results from both these points of view, but since the calculated weight of "fat" in the liver of the 100 g. rat reflects both these changes, it gives a more complete picture of the results. Accordingly, the results will be discussed only on the basis of the weight of the constituents in the liver of the 100 g. rat, although both the liver size and the percentages of "fat" are recorded in Tables II–V.

In Table II the extra choline intakes of groups 2 and 3 which received pancreatic extract, appear as negative values, because the food consumption of these groups was less than that of the others, the basal diets in both Exps. 1 and 2 being relatively rich in choline (32·5 mg. per 100 g.), since they contained marmite.

The results of groups 2 and 3 show that the administration of the extract derived from 0·44 and 0·08 g. of pancreas has lowered the amount of glyceride in the livers from the control figure of 1·96 g. to 0·88 and 1·11 g. respectively. These marked decreases show clearly the considerable effect of the pancreatic extract in preventing fat deposition in the liver. Further, this effect is not due to choline, for the choline intakes of these two groups were actually less than

Table II. *Exp. 1. Comparative activities of choline and pancreatic extracts on glyceride deposition in the "cholesterol" fatty liver*

Group	Supplement administered	Average initial body wt. g.	Wt. loss % initial body wt.	Food intake g.	Extract administered: Actual wt./rat/day mg.	Extract administered: Pancreas equivalent g.	Extra choline intake/rat/day mg.	Liver % final body wt.	Total lipoid: % fresh liver	Total lipoid: In liver of 100 g. rat g.	Glyceride: % fresh liver	Glyceride: In liver of 100 g. rat g.
1	—	180	6	9·0	—	—	—	6·3	37·3	2·34	31·3	1·96
2	Pancreatic extract	176	7	7·4	24·4	0·44	−0·3	4·7	24·6	1·14	19·0	0·88
3	Pancreatic extract	165	4	8·0	4·4	0·08	−0·3	5·0	29·2	1·46	22·1	1·11
4	Choline	167	2	9·1	—	—	9·2	4·0	13·9	0·54	7·4	0·29
5	Choline	167	2	8·5	—	—	4·1	4·6	20·3	0·93	13·7	0·63

Table III. *Exp. 2. Comparative activities of choline and pancreatic extracts on total fatty acid content of "cholesterol" fatty liver*

Group	Supplement administered	Average initial body wt. g.	Wt. loss % initial body wt.	Extract administered: Actual wt./rat/day mg.	Extract administered: Pancreas equivalent g.	Extra choline intake/rat/day mg.	Liver % final body wt.	Total lipoid: % fresh liver	Total lipoid: In liver of 100 g. rat g.	Fatty acids: % fresh liver	Fatty acids: In liver of 100 g. rat g.
6	—	143	0	—	—	—	5·9	30·9	1·83	25·1	1·48
7	Pancreatic extract	139	4	93	0·74	2·3	4·3	18·6	0·80	13·0	0·56
8	Pancreatic extract	161	4	46	0·37	1·2	4·7	22·8	1·07	17·3	0·81
9	Pancreatic extract	146	5	24	0·19	0·6	5·2	30·3	1·58	24·1	1·26
10	Pancreatic extract	154	6	9	0·07	0·2	5·5	34·4	1·89	28·8	1·58
11	Pancreatic extract	149	8	1	0·008	0·02	5·0	32·5	1·64	26·2	1·32
12	Choline	153	4	—	—	5·5	4·3	18·7	0·80	13·7	0·58
13	Choline	161	13	—	—	3·9	4·0	18·8	0·75	13·6	0·54
14	Choline	156	9	—	—	3·1	4·1	15·2	0·62	10·6	0·53
15	Choline	166	7	—	—	2·3	4·5	21·6	0·96	16·7	0·74
16	Choline	162	7	—	—	1·6	4·6	24·8	1·15	19·6	0·91
17	Choline	158	5	—	—	0·8	5·7	32·2	1·83	26·8	1·52

that of the control group. Since the greater decrease in group 3, 1·08 g., is less than the 1·33 g. decrease caused by 4·1 mg. choline in group 5, it is impossible accurately to assess the activity of the extract in terms of choline. If the arbitrary assumption be made that the preventive action of choline is linear over this range of liver fat percentages, then the extract from 0·44 g. pancreas has been as effective as $\frac{108}{133} \times 4{\cdot}1$ mg. choline. The pancreatic extract thus has an activity equivalent to 757 mg. choline per 100 g. pancreas, whereas its contained choline represents 55 mg. per 100 g. i.e. its activity is about 14 times that of its contained choline. While the assumption as to the linear nature of the effect is probably not strictly accurate, the results of this experiment appear conclusive in showing the presence in the pancreatic extract of an active substance other than choline. It is to be mentioned also that if the decrease in group 3 were equated with that of group 5 in the same manner as was done for group 2, the activity of the pancreatic extract would have appeared considerably greater even than that found above.

The results in Table III illustrate the limitations of the experimental method, and the caution which is necessary in interpreting the results. The livers of the control group contained 1·48 g. fatty acid; this decreases to 0·56 g. with the highest dose of extract (pancreas equivalent 0·74 g.) and with succeeding doses the amount increases until in group 10 with a pancreas equivalent of 0·07 g., it is 1·58 g., a figure very similar to that of the control group. In the last of the pancreatic groups, however, the amount falls to 1·32 g. yet clearly it should be identical with that of the control group, since the amount of the pancreas equivalent was negligibly small in this group. This illustrates the variations which are to be encountered even when groups of 10 animals are used and emphasizes the unreliability of attempts to equate the effect of any particular dosage of pancreatic extract unless it has a pronounced effect in decreasing the amount of liver fat.

The choline groups, 12–17, illustrate another difficulty, namely that it is not possible to demonstrate clearly changes caused by small successive decreases in the amount of choline administered, unless the doses employed are near the limiting effective amounts of 2–3 mg. Thus each animal of groups 12, 13 and 14 received daily 5·5, 3·9 and 3·1 mg. choline respectively; yet the amounts of fat in the livers of these three groups, 0·58, 0·54 and 0·53 g. are the same. It is thus impossible to differentiate between the effects of 3·1, 3·9 and 5·5 mg. choline. The decreasing effects of amounts of choline lower than these appear, however, in groups 15, 16 and 17, in the last of which with an intake of only 0·8 mg. the amount of fatty acids in the liver, 1·52 g., has returned to the control figure 1·48 g.

The results of pancreatic group 7, therefore, could be equated with those of either of the choline groups 12, 13 or 14. In order to obtain the minimum figure for the activity of the pancreatic extract it is considered wise to equate group 7 with group 14 although it must be emphasized that the effect of the pancreas may be underestimated by so doing. If this be done the extract from 0·74 g. pancreas and containing 2·3 mg. choline has an action equal to 3·1 mg. choline. A second value may be obtained from the results of group 8, where the liver fat is 0·81 g., which lie between those of groups 15 and 16 in which the amounts are 0·74 and 0·91 g. respectively. If an intermediate value be taken, a pancreas equivalent of 0·37 g. and containing 1·2 mg. choline is equivalent in action to 2·0 mg. choline. From the results of groups 7 and 8, therefore, the pancreatic extract in this experiment had an action equal to that of 419 and 541 mg.

Table IV. *Comparative activities of choline and pancreatic extracts on total "fat" content of "fat" fatty liver*

Group	Supplement administered	Average initial body wt. g.	Wt. loss % initial body wt.	Extract administered: Actual wt./rat/day mg.	Extract administered: Pancreas equivalent g.	Extra choline intake/rat/day mg.	Liver % final body wt.	"Fat" % fresh liver	"Fat" in liver of 100 g. rat g.
				Exp. 3					
18	—	154	12	—	—	—	4·7	21·7	1·07
19	Pancreatic extract	156	12	100	0·96	1·6	3·7	9·2	0·34
20	Pancreatic extract	155	9	80	0·77	1·3	3·8	10·9	0·42
21	Pancreatic extract	150	10	61	0·58	1·0	3·8	9·7	0·36
22	Pancreatic extract	158	14	40	0·38	0·65	4·4	12·2	0·55
23	Choline	157	12	—	—	10·1	3·4	5·0	0·17
24	Choline	156	14	—	—	7·4	3·8	7·2	0·26
25	Choline	155	14	—	—	5·4	3·6	7·7	0·28
26	Choline	152	13	—	—	3·4	4·0	10·1	0·41
				Exp. 4					
27	—	147	13	—	—	—	4·9	17·0	0·91
28	Pancreatic extract	141	12	50	0·48	0·82	4·4	13·3	0·60
29	Pancreatic extract	146	7	25	0·24	0·41	4·3	13·5	0·63
30	Pancreatic extract*	139	9	26	0·32	0·70	5·0	11·0	0·55
31	Choline	144	7	—	—	3·5	4·3	9·6	0·41
32	Choline	143	9	—	—	2·3	4·5	13·7	0·64
33	Choline	136	10	—	—	1·4	4·6	14·6	0·71
34	Choline	146	7	—	—	0·7	4·9	15·9	0·78
				Exp. 5					
35	—	158	3	—	—	—	5·3	16·3	0·98
36	Pancreatic extract	166	2	—	0·24	0·4	4·7	14·4	0·71
37	Choline	161	4	—	—	3·5	3·9	13·1	0·53
38	Choline	168	3	—	—	2·1	4·5	13·5	0·71

* Filtrate from precipitation with trichloroacetic acid.

Table V. *Exp. 6. Comparative activities of choline and pancreatic extracts on glyceride deposition in the "cholesterol" fatty liver*

Group	Supplement administered	Average initial body wt. g.	Wt. loss % initial body wt.	Extract administered: Actual wt./rat/day mg.	Extract administered: Pancreas equivalent g.	Extra choline intake/rat/day mg.	Liver % final body wt.	Total lipoids: % fresh liver	Total lipoids: In liver of 100 g. rat g.	Glyceride: % fresh liver	Glyceride: In liver of 100 g. rat g.
39	—	165	5	—	—	—	8·6	42·3	3·60	36·6	3·11
40	Pancreatic extract	178	7	19	0·40	0·6	6·2	30·66	1·89	24·7	1·53
41	—	167	7	3·7	0·08	0·1	6·5	35·8	2·38	29·8	1·98
42	Choline	164	5	—	—	2·1	5·9	31·6	1·85	25·7	1·51

choline per 100 g. pancreas, while its actual choline content corresponded to 311 mg. Its activity is thus 1·3 or 1·7 times that of its contained choline.

The impossibility of differentiating between the effects of different doses of a supplement unless they differ significantly in amount, which was discussed for groups 12–14 of Exp. 2, is again illustrated in Exp. 3 by groups 19–21 receiving pancreatic extract and groups 23–25 receiving choline. In order not to favour the pancreatic extract choline group 26 is equated with group 20, even though the amount of fat is lower in group 21, which received a smaller pancreas equivalent. Calculating as previously described, the pancreatic extract of Exp. 3 has an action per 100 g. pancreas equal to 442 mg. choline as compared with its content of 171 mg.

The extract used for Exp. 4 was the same as that of Exp. 3. In addition group 30 received the material obtained from the filtrate after precipitating the extract with trichloroacetic acid as described earlier. Both the pancreatic groups 28 and 29 may be equated against the choline group 32. If this be done the extract has an activity equivalent to 479 or 958 mg. choline per 100 g. pancreas. The former of these two amounts is similar to the 442 mg. found for this particular extract in Exp. 3 as described above. These two results, one twice as great as the other, illustrate the limitations of the method when applied to small groups of 10 animals. The trichloroacetic acid filtrate of the pancreatic extract appears to retain all the original activity, for group 30, when equated against group 32, gives an activity equal to that of 712 mg. choline. For the pancreatic extract used in Exps. 3 and 4 the non-choline activity found is thus 442, 479 and 958 mg. choline, and for the trichloroacetic acid filtrate 712 mg.

In Exp. 5, group 36, receiving pancreatic extract, has given a result identical with that of the choline group 38, corresponding to 875 mg. choline as compared with its choline content of 180 mg. During this experiment two other groups of animals received the material of the trichloroacetic acid filtrate obtained by direct extraction of the original pancreas after removal of the trichloroacetic acid as described for Exp. 4. The results which are not recorded in the tables suggested that this method of direct extraction of the pancreas was not as effective as the alcoholic extraction used in the other experiments.

In Exp. 6, pancreatic group 40 may be equated with choline group 42. The activity of this extract is thus 525 mg. choline per 100 g. pancreas; its choline content was 153 mg.

Discussion

Data are presented in Table VI which emphasize the more important points which need discussion. The weight of pancreas equivalent to the volume of extract administered and the actual solids of the ingested extract are recorded as before. Column (*c*) records in terms of choline the calculated non-choline activity of the pancreatic extract administered, and is obtained by subtraction of the "extra choline intake" of the pancreas groups of Tables II–V from the particular choline dosage with which each extract dosage was equated. Column (*d*) then expresses the non-choline activity of each extract referred to 100 g. pancreas, expressed in terms of mg. choline.

During the six experiments separate extracts were used in Exps. 1, 2, 5 and 6; a fifth extract was used both for Exps. 3 and 4, and also a trichloroacetic acid filtrate of this extract in Exp. 4. As shown in Table VI, it is possible to assess the activity of the five extracts in terms of choline on eight occasions. After allowing for the actual choline contents of the extracts, which had been previously determined in every case, all the extracts had an action in preventing

Table VI. *Assessment of the activity of pancreatic extracts in terms of choline*

Exp.	Extract administered: Pancreas equivalent/rat/day g. (*a*)	Extract administered: Solids mg. (*b*)	Choline equivalent of the non-choline activity of the pancreatic extract administered mg. (choline) (*c*)	Non-choline activity of extract/100 g. pancreas mg. (choline) (*d*)
1	0·44	24	3·3	757
2	0·74	93	0·8	108
	0·37	46	0·8	216
3	0·77	80	2·1	273
4	0·48	50	1·5	308
	0·32*	26	2·1	670
5	0·24	—	1·7	704
6	0·40	19	1·5	375

* Filtrate from precipitation with trichloroacetic acid.

fat deposition in the liver varying from an equivalent of 108 mg. in Exp. 2 to 757 mg. in Exp. 1, when expressed as mg. choline per 100 g. pancreas (column (*d*), Table VI).

The method of assay is not as accurate as could be desired but care has been taken in each case to assess the activity of the pancreatic extracts at a minimum. For example, in Exp. 2, group 7 was equated with group 14, giving a non-choline activity of 108 (Table VI). Groups 12 and 13 however both gave the same amount of fat in the liver as group 14, and had group 7 been equated with either of these, the non-choline activity would have been assessed as 432 and 216 mg. choline per 100 g. pancreas, amounts four times and twice as great. Similarly, if in Exp. 3 group 21 and not group 20 had been equated with choline group 26, the activity found would have been 586 mg. instead of 442 mg. choline per 100 g. pancreas. The average value for the non-choline activity in the eight assays of the 5 extracts corresponds to 426 mg. choline per 100 g. pancreas. It might be objected that since the food intakes were not measured save in Exp. 1, only the results of that experiment are valid, for if the choline groups consumed less food than the 8 g. daily on which the assays are based, clearly the activities of the pancreatic extracts would be assessed at too high a figure. This objection cannot be sustained. In the first place, the food intakes of the choline groups in Exp. 1 are higher than those of the pancreatic groups, and it has been our general experience that these fatty diets are better consumed when choline supplements are added. Secondly the food intakes of the choline groups necessary to reduce the non-choline activity of the extracts to zero, may be calculated. Such calculations show that the amounts would have to be so low as 5·9 and 4·8 g. in Exp. 2, 3·1 g. in Exp. 3, 2·8 and 1·4 g. in Exp. 4, 1·5 g. in Exp. 5 and 2·3 g. in Exp. 6. Since the weight losses of the choline groups were similar to those of the pancreas groups, such values for food intake are clearly impossible. We think it probable that the assumption of a uniform food intake for all the groups has in fact operated against the pancreas rather than in its favour, as it would have done had it been made in Exp. 1.

It is of interest to calculate the percentage of the total activity of each extract which is due to its choline content. This value ranges from 0 % in Exp. 1 to 74 % in Exp. 2 with a mean value of 35 %. Thus the choline content of the

extracts accounts for only one-third of their activity. Since this is, as already pointed out, a minimum estimate, the presence of a non-choline factor in pancreas appears established, thus extending the claims of Dragstedt *et al.* [1936, 1, 2] to the dietary fatty liver.

While the *non-choline* activity of the extracts, 426 mg., is about 1½ times the actual choline content of the whole pancreas, it is to be admitted that this represents a low degree of activity if the substance is a hormone. The probable explanation is that the method of extraction is inefficient, but in view of the conflicting evidence now to be reviewed, we thought it better to establish the presence of the substance before attempting to improve its extraction.

Since proteins exert a preventive action in liver fat deposition [Channon & Wilkinson, 1935; Beeston *et al.* 1935; Best *et al.* 1936; Channon *et al.* 1938], it is possible that the extra activity of the extracts may be due to their content of protein. In Table I it is shown that the solids of the extracts of Exps. 1 and 2 contained 10·9 and 11·8% total N, and 8·0 and 10·8% non-protein-N respectively. In Exp. 1 the protein-N was, therefore, 2·9% corresponding to 18·1% of protein, and of the 24 mg. actual solids administered 4·3 mg. were protein. If the activity were due to protein it may be calculated that the protein would possess an activity equal to 767 mg. choline per g. Similar calculation shows that in Exp. 2, 1 g. of protein would have the activity of 138 mg. choline. Of many proteins studied [Channon *et al.* 1938] none has an activity significantly greater than that found by Beeston *et al.* [1936] for caseinogen, i.e. 7 mg. choline per g. Clearly, therefore, a protein cannot be responsible for the non-choline effect of the pancreas unless it possesses an activity vastly greater than that of any yet encountered. Further in a single observation in Exp. 4 the trichloroacetic acid filtrate was found to retain all the original activity of th extract. It must not be overlooked, however, that the active substance may have been present in the pancreas originally as part of a protein molecule and liberated by enzymic action during the earlier stages of preparation of the extracts.

The work of Dragstedt and his colleagues has been much criticized, and failure to repeat his results both on depancreatized dogs and on rats has been reported by a number of workers. Chaikoff & Kaplan [1937] have drawn attention to the impossibility of making comparative studies of the effects of choline and pancreatic extracts in any quantitative manner by the histological methods which Dragstedt employed. They have further shown that the fat distribution in the livers of depancreatized dogs varies from lobe to lobe, and therefore that results based on histological examination of biopsy samples are unreliable. Using depancreatized dogs, Kaplan & Chaikoff [1937] were not satisfied that pancreatic extracts had an effect other than that of their choline content. They point out that until properly designed experiments have been carried out in which the choline contents of the extracts are determined and taken into account in any assays, the results of Dragstedt *et al.* [1936, 2] cannot be accepted.

Aylward & Holt [1937] fed groups of rats on a diet causing fatty livers; other groups received the same diet with the addition of raw pancreas or corresponding amounts of choline calculated from the figures for ox pancreas recorded by Fletcher *et al.* [1935]. They concluded that the effect of raw pancreas was adequately explained by its choline content. Study of their results, however shows that the amounts of pancreas and choline fed were such that the "fat" percentage in the livers was in our opinion only sufficiently high at one pancreas dosage for any adequate comparison to be made between the effects of the two

supplements; in addition they based their conclusions on the percentage of fat in the livers only and took no account of the changes in liver size. In this one result, calculation from their data of the amount of fat in the liver of the 100 g. rat shows that 1 g. of pancreas had an action approximately equal to that of 3·5 mg. choline which may be contrasted with the 2·3 mg. choline per g. of ox pancreas reported by Fletcher *et al.* [1935]. Owing to the possible lipotropic effect of the proteins of the pancreas fed, this single result provides but slight evidence as to the existence of an active pancreatic factor other than choline, especially since raw pancreas was fed, and any active non-choline compound in it may have been inactivated by enzyme action. MacKay [1937] administered extracts prepared by Dragstedt's method to rats receiving a diet causing fatty livers in amounts equivalent to 1·2–30 g. of pancreas per rat per day. The livers of the control animals contained 21·5 % fat, and no significant change occurred until the animals received the equivalent of 3 g. of pancreas per day, when it fell to 13·2 %. The author deduced that the pancreatic extract was active in preventing fat deposition, and, holding the view that the extract could not contain enough lecithin or choline to give the experimental results quoted, attributed its action to a non-choline factor. As shown in Table I, however, such extracts may contain much choline, and indeed in a later paper [MacKay & Barnes, 1938], the same worker attributes half the activity of such extracts to their contained choline. Since the choline contents of the extracts were not determined, while in addition the extracts from very considerable amounts of pancreas were found necessary in order to demonstrate an effect, it is difficult in our opinion to draw any conclusions from these results. MacKay & Barnes [1938] reported further experiments in which extracts equivalent to very much smaller amounts of pancreas (up to 1 g. per rat per day) were found active. Finding an approximately equal distribution of activity in the filtrate and precipitate obtained by the addition of 20 vol. of alcohol to their extract, they regarded this as evidence that both the choline and the protein present in pancreatic extracts act to prevent fat deposition in the liver. In contrast to the previous finding [MacKay, 1937] they concluded that there is no specific pancreatic substance, the action of such extracts being the sum of the activities of the choline and protein. Since the crude alcohol precipitate was regarded as protein, and the choline content of neither fraction was estimated, this deduction appears unwarranted. Further, the liver fat percentages in the control groups of two of their three experiments, 8·2 and 7·0, are in our experience far too low to enable any adequate demonstration of the activity of a supplement to be made.

The only existing claim of confirmation of Dragstedt's results occurs in a preliminary note by Shapiro & Wertheimer [1937]. In this work each rat received extract, of undetermined choline content, equivalent to 14–22 g. pancreas per day, in contrast with the maximum of 0·9 g. used in the present work. From diffusion and adsorption experiments, the authors present some evidence which may be taken as indicating the non-identity of choline itself with the active substance.

For the reasons outlined above, it appears to us that the work which has so far been reviewed neither proves nor disproves the existence of a pancreatic non-choline factor influencing liver fat.

Quite recently Dragstedt *et al.* [1938] have adduced further evidence in confirmation of their original findings.

Best & Ridout [1938] have just reported experiments in which they used a pancreatic extract proved active on depancreatized dogs by Dragstedt; their

results lead them to the conclusion that "lipocaic" has no action on rats beyond that to be expected from its content of choline and protein, and that its activity is no more than that of an equal weight of dietary caseinogen, namely 5 mg. choline per g. From the data presented in this paper, values for the lipotropic value of the solids of our extracts may be calculated: such values range from 33 to 138 mg. choline per g. with an average of 72 mg. per g., which is 14 times that accepted by Best & Ridout [1938] for caseinogen. Since our extracts contained appreciable amounts of choline while the "lipocaic" used by Best & Ridout [1938] contained only traces, it would be fairer to calculate the non-choline lipotropic values; these vary from 9 to 138 mg. per g. and average 54 mg. per g., a figure 10 times that found by the Toronto workers. Their opinion is thus in marked and perplexing contrast to that presented in this paper, and in view of the very considerable body of consistent evidence which we offer, we feel that the explanation of the divergence must await further experiment. The only suggestion we offer is that possibly the single preparation of "lipocaic" administered by Best & Ridout [1938] to each of the two groups of rats used had become inactive subsequent to its being tested by Dragstedt; for this reason it would have been of value had it been possible for the preparation after being found inactive on rats at Toronto, to be re-tested for its activity on depancreatized dogs by Dragstedt.

If the conclusions of Best & Ridout [1938] and of Dragstedt *et al.* [1936, 2] are confirmed they will provide a further example of differences between the factors influencing the dietary fatty liver and those affecting the diabetic fatty liver. One such difference is indicated by the original results of Allan *et al.* [1924] and by numerous later studies; the high protein diets on which depancreatized dogs are maintained fail to prevent the development of fatty livers in these animals, yet it is the protein content of diets low in choline which controls the degree of fat infiltration in the rat [Channon & Wilkinson, 1935; Beeston *et al.* 1935; Best *et al.* 1936; Channon *et al.* 1938]. It would, however, be a curious fact if in diabetic dogs developing fatty livers on diets of high protein content, the addition of raw pancreas controlled such fat infiltration by virtue of the additional protein provided, unless the pancreatic protein is of specific nature: alternatively the pancreas must contain some active non-protein substance other than choline.

Summary

1. Dilute alcoholic extracts of pancreas have been prepared according to the method of Dragstedt *et al.* [1936, 2] and the amount of choline in them determined. The action of each extract in preventing fat deposition in the livers of rats has been assayed in terms of choline.

2. Six experiments were carried out on 42 groups of rats, each group consisting of 10 animals. Three of these experiments were concerned with the "cholesterol" and three with the "fat" fatty liver.

3. Eight assays of the activity of the five pancreatic extracts used were made; all of them showed the extracts to possess an ability to prevent fat deposition in the liver greater than could be attributed to their content of choline. The average value for this non-choline activity was equivalent to that of 426 mg. choline per 100 g. pancreas while the choline present in the extracts accounted for only one-third of their activity.

4. The non-choline activity is not accounted for by the protein content of the extracts and it is concluded that there exists in pancreas a substance other than choline which is involved in fat deposition in the liver.

5. Some of the difficulties of assaying pancreatic extracts are pointed out, and the conflicting evidence on the problem of the activity of such extracts is discussed.

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CLXXVIII. THE EFFECT OF CHOLESTEROL FEEDING ON LIPOID DEPOSITION IN THE LIVER OF RATS

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When rats are fed on diets containing cholesterol, they develop fatty livers which are characterized by the presence in the liver of excessive amounts of cholesteryl esters, while at the same time considerable deposition of glyceride may occur. This variable increase in the glyceride content of the "cholesterol" fatty liver has been shown to depend on a number of factors, such as the dietary contents of choline [Best *et al.* 1934], of caseinogen [Channon & Wilkinson, 1935; Beeston *et al.* 1935], of methionine [Channon *et al.* 1938] and of certain choline analogues [Channon *et al.* 1937, 1, 2]. While the nature of the "cholesterol" fatty liver and the effects on its composition of a number of dietary and other factors have thus been considerably studied, the part played by the fat content of the diet has not been defined. The work described in the present paper has been designed to investigate the quantitative changes which occur in the "cholesterol" fatty liver, as a result of variations in the fat content of the diet, with particular reference to the deposition of cholesterol and of glyceride on diets of constant cholesterol content.

Experimental

The general method of experiment was to feed groups each of 10 rats on diets containing varying amounts of beef dripping with and without 2% cholesterol. In Exp. 1 all the animals were does and the period of feeding was 21 days. The four control groups received a diet consisting of caseinogen (alcohol-extracted) 8, marmite 5, salt mixture 5, cod liver oil 1, with 10, 20, 30 and 40 parts of beef dripping respectively, each diet being made up to 100 parts with glucose. Four other groups received similar diets with the substitution of 2 parts of cholesterol at the expense of glucose. The food intake of the animals was not measured. After 21 days the animals were guillotined and the pooled livers of each group were treated by the alcohol-ether method, and analysed for phosphatide, glyceride and free and combined cholesterol [Best *et al.* 1934]. In Exps. 2 and 3 control groups were omitted. In Exp. 2 the cholesterol-containing diets were identical with those of Exp. 1 save that the caseinogen content of the diet was decreased to 5% and the glucose correspondingly increased. The animals were bucks and the duration of the experiment 17 days. The livers of the animals were investigated individually, and the amount of the fatty acids and unsaponifiable matter determined together by the saponification method. The materials obtained from the livers of the animals of each group were then combined and analysed for their content of unsaponifiable matter, the fatty acids being obtained by difference. In group 12 (Table II) three animals in which the weight losses were greater than 20% were rejected. In Exp. 3 the

diets were the same as for Exp. 2, save that the fat contents of the four diets were 5, 15, 25 and 35 parts respectively, the glucose being correspondingly increased. The animals (♂♂) were fed for 21 days, and the pooled livers were worked up by the alcohol-ether method; the total ethereal extract was analysed for fatty acids and unsaponifiable matter only, the sterol content of the latter being determined by the digitonin method. The essential data are recorded in Tables I–III, in which the amounts of the different constituents are recorded both as percentages of the fresh liver weight and as the weight in the liver of the 100 g. rat.

Results

Since the control groups, 1*a*–4*a* of Exp. 1, provide data concerning the effect of progressive increases in the fat content of the diet on the nature of the resulting fatty liver, they will be discussed together. Mention is first necessary of findings with normal animals. Work of the last few years has shown that an increasing number of dietary factors are concerned in the control of liver fat. The consequent growing realization of the complexity of liver fat control makes it uncertain as to what should be regarded as "normal" values. For comparison with groups 1*a*–4*a*, the liver of the "normal" animal on a balanced mixed diet is regarded as being 3·4 % of the body weight and as containing lecithin 3·3, cholesterol 0·25–0·30, cholesteryl esters 0·03 and glyceride 1·0 % of the fresh liver weight. For the 100 g. rat, these latter figures entail the presence in the liver of lecithin 111, cholesterol 8–10, cholesteryl oleate 1 and glyceride 34 mg. (total fatty acids 112 mg.).

Table I shows that increase in the fat content of the diet causes in general increases in the size of the liver, although these increases do not run strictly parallel with the increasing fat content. Similarly the phosphatide figures show decreases in the percentages of lecithin with increasing fat infiltration, although group 3*a* contains less lecithin, 2·30 %, than group 4*a*, 2·63 %. On the basis of the 100 g. rat, however, the livers contain similar amounts of phosphatide 112, 114, 103 and 105 mg. respectively, and these do not differ sufficiently from the normal 111 mg. to be of significance. The percentage of free cholesterol, 0·21, is constant throughout, while the increase in liver size is not sufficiently great significantly to increase its absolute amount. More pronounced changes occur in cholesteryl ester fractions which increase from the normal 0·03 % and 1 mg. to a maximum of 0·33 % and 14 mg. The most striking changes occur, however, in the glyceride fractions which show successive increases from 4·10 to 17·54 % (normal 1 %) as the fat content of the diet is progressively increased. The absolute weight in the liver of the 100 g. rat thus progressively rises to a maximum of 0·722 g., the amounts present in the 11, 21, 31 and 41 % fat groups respectively being about 4, 9, 15 and 21 times greater than the normal. Noteworthy also is the fact that the glyceride fraction whether expressed as a percentage of the liver or in terms of absolute weight, shows a linear increase with the amount of fat in the diet.

The results of the cholesterol groups 1*b*–4*b* will now be compared with those of the control groups just discussed. In all cases the liver weight is increased considerably in the cholesterol groups compared with the corresponding control groups. The greatest increase is shown in group 4*b* in which the liver constitutes 6·88 % of the body weight, a 70 % increase over the control group 4*a*, 4·12 %. While the inclusion in the diet of 2 % cholesterol thus has a profound effect on the liver size at all levels of dietary fat, among themselves the cholesterol

groups do not increase regularly in this respect, for group 3*b* at 4·97 % is lower than group 2*b*, 5·24 %. In the control groups 3*a* and 4*a* a similar inconsistency in this matter of liver size is shown. In every case also the percentages of lecithin are lower than those of the control groups, the extremely low figures of 1·62 and 1·14 % occurring in groups 3*b* and 4*b*. While the actual weights of lecithin in groups 1*b* and 2*b*, 118 and 115 mg. respectively, are normal, they are significantly decreased in the former groups to 81 and 77 mg. It has usually been found in this laboratory in experiments on the "cholesterol" fatty liver, that the absolute amounts of lecithin are unchanged with progressive infiltration. Thus Beeston *et al.* [1935] carried out four experiments, in the control groups of which the total weight of lipoids in the livers varied from 1·720 to 0·618 g. Reduction of these amounts to 0·281 and 0·528 g. respectively by large doses of choline (about 75 mg. per rat per day) had no effect in increasing the absolute weight of lecithin in the liver although the percentages were of course increased. Similarly in other experiments which provided considerable data on this point [Channon *et al.* 1937, 1, 2] the same result held. The results with groups 3*b* and 4*b* are thus contrary to the general experience in this laboratory.

The effect of the inclusion of cholesterol in the diets is most marked on the cholesterol and glyceride fractions. The free cholesterol shows increases to percentage figures which are 25–50 greater than those of the control groups, while the actual weight in the livers is approximately doubled. These changes are, however, small compared with those in the cholesteryl ester fractions, which increase from the control figures of 0·14, 0·32, 0·31 and 0·33 % in groups 1*a*–4*a* to 1·88, 2·44, 3·14 and 3·86 % in the corresponding cholesterol groups. The actual weights of cholesteryl ester in the liver of the 100 g. rat thus pass from 0·071 to 0·265 g., amounts which are 14, 11, 11 and 18 times greater than those of the corresponding control groups. The amounts of glyceride in groups 1*b*–4*b* also provide clear evidence of the influence of cholesterol in increasing the glyceride fraction of the liver. In all cases, the percentages of glyceride present in the livers of the cholesterol-fed animals are much greater than in the control groups, the minimum increase being in group 1*b* in which it is increased by 10·33 units over group 1*a*, and the maximum being in group 2*b* in which it is 20·3 units % greater than in its control group 2*a*. It might have been anticipated that the amounts of glyceride in the cholesterol-fed animals would progressively increase as the fat content of the diet increased. This, however, is not the case, for although the percentages and amounts of liver glyceride in groups 2*b* and 3*b* lie between those of groups 1*b* and 4*b*, they are not in ascending order. Since in the control groups the amounts of liver glyceride show a linear relationship to the fat content of the diet and in the cholesterol groups the cholesterol contents of the livers do likewise, it seems improbable that group variations are responsible for these irregularities. Accordingly two further experiments were carried out with the object of obtaining further evidence concerning the extent of the deposition of glyceride caused by cholesterol feeding in diets of increasing fat content, and secondly to confirm the progressive increases in cholesterol deposition in the liver observed in Exp. 1. Since both these objects appeared to be obtainable without the use of control groups no such groups were used in Exps. 2 and 3. Before presenting the results of Exps. 2 and 3 in Table II, one point needs mention. In Exp. 2 the amount of cholesterol present in the unsaponifiable fraction of the livers was not determined. Since in cholesterol-fed animals cholesterol constitutes some 90 % of the liver unsaponifiable fraction, the extreme limits in Exp. 3 being 88–92 %, for the purpose of this discussion the unsaponifiable fraction will be regarded as cholesterol. The amount of free cholesterol in the

Table I. *Exp. 1. The effect of the inclusion of cholesterol on liver lipoids in diets of varying fat content*

Group	Fat in diet %	Cholesterol in diet	Liver (% body wt.)	Liver lipoids (g. per 100 g. fresh liver) Lecithin	Cholesterol	Cholesteryl oleate	Glyceride	Total	Liver lipoids (g. per liver of 100 g. rat) Lecithin	Cholesterol	Cholesteryl oleate	Glyceride	Total
1*a*	11	—	3·62	3·11	0·21	0·14	4·10	7·56	0·112	0·007	0·005	0·148	0·272
1*b*	11	2	4·00	2·95	0·32	1·88	14·43	19·58	0·118	0·014	0·071	0·577	0·780
2*a*	21	—	3·84	3·05	0·21	0·32	8·25	11·83	0·114	0·008	0·012	0·317	0·451
2*b*	21	2	5·24	2·19	0·27	2·44	28·55	33·45	0·115	0·014	0·128	1·496	1·753
3*a*	31	—	4·49	2·30	0·21	0·31	11·22	14·04	0·103	0·010	0·014	0·505	0·632
3*b*	31	2	4·97	1·62	0·28	3·14	24·14	29·18	0·081	0·014	0·156	1·191	1·442
4*a*	41	—	4·12	2·63	0·21	0·33	17·54	20·71	0·105	0·009	0·014	0·722	0·850
4*b*	41	2	6·88	1·14	0·28	3·86	34·40	39·68	0·077	0·019	0·265	2·367	2·728

Table III. *Exp. 3. The effect of the inclusion of cholesterol on liver lipoids in diets of varying fat content*

Group	Fat in diet %	No. of rats	Wt. gain or loss %	Liver % body wt.	Liver lipoids: g. per 100 g. fresh liver Fatty acids (*a*)	Unsap. matter (*b*)	Cholesterol	Total (*a* + *b*)	Liver lipoids: g. per liver of 100 g. rat Fatty acids (*c*)	Unsap. matter (*d*)	Cholesterol	Total (*c* + *d*)
13	6	10	−4·7	4·00	15·94	2·49	2·21	18·43	0·638	0·096	0·085	0·734
14	16	10	−4·7	4·00	17·70	3·01	2·59	20·71	0·709	0·120	0·103	0·829
15	26	10	−8·7	4·37	24·70	3·26	2·83	27·96	1·078	0·140	0·122	1·218
16	36	10	−5·7	4·96	25·37	3·78	3·29	29·15	1·284	0·192	0·167	1·476

"cholesterol" fatty liver being small and subject to relatively little change, changes in total cholesterol represent substantially changes in cholesteryl esters. The results are presented in Tables II and III.

Table II. *Exp. 2. The effect of the inclusion of cholesterol on liver lipoids in diets of varying fat content*

					Liver lipoids					
					g. per 100 g. fresh liver			g. per liver of 100 g. rat		
Group	Fat in diet %	No. of rats	Wt. gain or loss %	Liver % body wt.	Fatty acid	Unsap. matter	Total	Fatty acid	Unsap. matter	Total
9	11	10	− 7·9	4·20	14·04	1·61	15·65	0·588	0·072	0·660
10	21	10	−10·4	3·94	14·23	2·75	16·98	0·543	0·106	0·649
11	31	10	− 9·7	5·88	29·76	2·35	32·11	1·748	0·135	1·883
12	41	7	−11·1	5·81	29·63	2·85	32·48	1·732	0·156	1·888

The results presented in Table II call for comment on two aspects only, for in general they confirm those of Exp. 1. First, with regard to the cholesterol content of the liver, the percentage in Exp. 2 rises from 1·61 on the diet containing 11 % fat to 2·85 on that with 41 % fat. The percentages in groups 10 and 11, while lying between those of groups 9 and 12, are not however in ascending order. In Exp. 3 the cholesterol increases regularly from 2·21 % in group 13 (6 % fat) to 3·29 in group 16 (36 % fat). In spite of the irregularity of the percentage figures in groups 10 and 11 in Exp. 2, the actual weights of cholesterol in the livers show again progressive increases in both experiments, from 0·072 to 0·156 g. in Exp. 2 and from 0·085 to 0·167 g. in Exp. 3. These amounts include of course the free cholesterol present, which, as shown in Exp. 1, is about 10 mg. The results of Exps. 2 and 3, therefore, confirm the finding of Exp. 1 that on diets of constant cholesterol but of increasing fat content the amount of cholesterol appearing in the liver is directly related to the fat content of the diet.

Considering now the fatty acid content, Table II shows that in Exp. 2 the four groups fall into pairs, i.e. groups 9 and 10 with 11 and 21 % fat in the diet contain 14·04 and 14·23 % fatty acids while groups 11 and 12 with 31 and 41 % fat in the diet contain 29·76 and 29·63 % fatty acid in the liver. The actual weights of fatty acids in the livers behave similarly. In Table III, Exp. 3 shows a similar phenomenon as regards the percentage figures, for groups 13 and 14 have 15·94 and 17·70 % fatty acid in the liver, while groups 15 and 16 contain 24·70 and 25·37 % fatty acid. The actual weights of fatty acid in this experiment, however, show a progressive increase with increasing dietary fat, although this is not as regular as in the control groups of Exp. 1.

Discussion

The results of the control groups in Exp. 1 show that increasing the fat content of the diet causes linear increases in the percentage and amount of glyceride, a decrease in the percentage but no change in the amount of phosphatide and a small but definite accumulation of cholesteryl esters. The question arises whether the changes in cholesteryl esters are metabolically significant. Previous work has shown that the inclusion of even small amounts of cholesterol in diets results in an increase in the amount of this substance in the liver. Thus Channon & Tristram [1937] found that the daily intake by the 100 g. rat of only 2·82 mg. cholesterol for 21 days caused about a 30 % increase in the total cholesterol of the liver, while 9·46 mg. caused a 70 % increase. Further, this

deposition of cholesterol as ester, may be detected within a few hours of feeding and is progressive with time, while little change takes place in the free sterol [Aylward *et al.* 1935]. For these reasons it seemed possible that the increases of cholesteryl ester caused by the increasing fat content of the control diets 1*a*–4*a* might be related to the cholesterol content of the beef dripping used in the diets rather than to the changes consequent on the accumulation of glyceride in the liver. While the cholesterol content of the actual fat used in these experiments was not determined, another sample of beef dripping was found to contain 0·2 % cholesterol. Every increase of 10 % in the fat content of the diet would thus result in the cholesterol intake being increased about 1 mg. per 100 g. rat per day, an amount which appears too small to account for the differences observed between group 1*a* and the other control groups. Work by Minovici [1935] and Eckstein & Treadwell [1935–6] indicates that diets containing unsaturated fatty acids promote sterol synthesis. This finding, if confirmed, may explain the deposition in the liver of small quantities of cholesteryl esters caused by high fat diets, low in lipotropic factors.

With regard to the results of the inclusion of 2 % cholesterol in the diet all three experiments show consistently that the amount of cholesteryl ester appearing in the liver is progressively increased by the fat content of the diet. The simplest explanation of this finding would be that the amount of cholesterol absorbed depends on the amount of fat being absorbed at the same time. Evidence on this question is, however, somewhat scanty and inconclusive. That cholesterol is not significantly absorbed unless fat is present in the diet is shown by the results of Thannhauser [1923] with human beings, Sano [1924] with dogs and Cook [1936] with rats. Some evidence exists also that absorption does not depend on the fat content of the diet. Chanutin & Ludewig [1933] fed two groups of rats on diets both of which contained 2·5 % cholesterol and 4 % cod liver oil, one diet containing 75 % carbohydrate and the other 75 % fat. Surprisingly, the amount of cholesterol in the livers of the animals on the high carbohydrate diet was 6 %, some 4 times greater than that in the livers of the animals receiving 75 % fat in the diet. Cook [1937] found no significant difference in the cholesterol contents of the livers of three groups of rats receiving 2 % cholesterol with 15, 20 and 30 % arachis oil in their diets respectively. The clear contrast between the present results and those of the latter author may perhaps be connected with the chemical nature of the dietary fat used.

One experiment was carried out in which the degree of absorption of cholesterol on a diet containing 40 % fat and 2 % cholesterol was measured. The cholesterol intake was calculated from the daily food intake and the analysis of the faeces of two groups, each consisting of eight animals kept in individual cages, and fed on the same diet with and without 2 % cholesterol. The average cholesterol intake was 132 mg. and the average excretion was 63 mg. per 100 g. rat per day. This shows a daily absorption of about 70 mg. per 100 g. rat which is nearly twice as great as the constant absorption of 0·3–0·4 g. per kg. observed by Cook [1937] in his three groups of animals referred to above. Whilst the accuracy of estimations of the degree of absorption of a substance such as cholesterol by the intake and excretion method is limited, it seems certain that greater absorption occurred in this experiment with 40 % fat, than in that of Cook [1937] with 15, 20 and 30 % arachis oil. It would have been desirable to measure the cholesterol absorption at a series of levels of fat in the diet, but it was not possible to pursue this study further, and until further evidence is available, the progressive increase in the liver cholesterol with increasing fat in the diet must be regarded as the result of increased cholesterol absorption.

It is possible by the addition of supplements of either choline [Best *et al.* 1934] or some of its analogues [Channon *et al.* 1937, 1, 2] or by increasing the protein content of the diet [Beeston *et al.* 1935] to produce "cholesterol" fatty livers containing amounts of glyceride only a few times greater than the normal. It appears reasonable, therefore, to believe that the amount of glyceride present in the liver does not control the deposition of cholesteryl esters. The converse of this does not, however, hold, for the results reported in this paper show that increase in the amount of cholesteryl ester in the liver consequent upon the inclusion of 2% cholesterol in appropriate diets, is the cause of the marked increase in the amount of the liver glyceride. This increase is apparent at all dietary fat levels, even when the glyceride content of the liver is already high. Thus in the control group, 4*a*, the amount of glyceride, 0·722 g., is some 21 times the normal, yet this is further increased to 70 times normal in group 4*b*, and similar results occur in the other groups.

Summary

1. The effect of varying the amount of dietary fat on the lipoids of the "fat" and "cholesterol" fatty livers has been investigated.

2. On a diet causing "fat" fatty livers in rats, the degree of glyceride infiltration is proportional to the amount of fat in the diet. There is a small increase in the amount of cholesteryl esters which does not appear to be related to the percentage of fat in the diet.

3. On a diet causing the "cholesterol" fatty liver, successive increases in the percentage of fat in the diet cause progressive and large increases in the cholesteryl ester content of the liver. Very considerable increases in the amounts of glyceride also occur, and the "cholesterol" fatty liver always contains much more glyceride than the "fat" fatty liver at any given level of dietary fat.

4. Small increases in the amounts of free cholesterol also occur with increases in the amount of dietary fats, and these become greater when cholesterol is present in the diets.

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CLXXIX. INVESTIGATIONS ON THE NATURE OF HAEMOPOIETIN, THE ANTI-ANAEMIC PRINCIPLE IN HOG'S STOMACH

IV. ON THE BIOCHEMICAL METHOD OF LASCH FOR THE QUANTITATIVE DETERMINATION OF "INTRINSIC FACTOR" IN GASTRIC JUICE

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(*Received 28 June 1938*)

Lasch [1937] has proposed a method for the early diagnosis of pernicious anaemia based on a test for the enzymic "intrinsic factor" in gastric juice. The extreme importance of such a test for the study of this enzyme and for early diagnosis led us to repeat it under the conditions employed by Lasch and we consider it desirable, at this stage, to record our findings.

The basis of Lasch's test is the hydrolysis of dry preparations of washed protein (beef muscle, liver, fibrin or yeast) by the action at *p*H 5·5–6 of the enzyme supposed to be present in normal gastric juice. The pepsin is removed by precipitation with caseinogen according to the method of Castle *et al.* [1931]; at the same time, Lasch argues that at *p*H 5·5, pepsin is in any case inactive. Normal gastric juice and the juices of patients showing achylia gastrica, excluding pernicious anaemia and carcinoma of the stomach, were claimed to have caused hydrolysis of the protein, but in the two last-named diseases proteolysis was said to be either absent or much reduced.

By incubating beef myoglobulin with normal gastric juice and juice from patients with pernicious anaemia and achlorhydric secondary anaemia at *p*H 6, Griffiths [1934] claimed to have obtained proteolysis with the normal juice only. This has since been criticized by Emerson & Helmer [1936] who showed that the small changes in non-protein nitrogen at *p*H 6 could have been due to the prolonged action of pepsin surviving irreversible inactivation. Taylor *et al.* [1937] showed that at *p*H 7·4 under conditions in which the possible actions of pepsin, trypsin and erepsin were excluded, normal gastric juice produces progressive liberation of non-protein nitrogen from caseinogen. They, however, remark that their experiments furnish no proof that this proteolysis is due to the interaction of the intrinsic factor with the caseinogen.

We have carried out the test described by Lasch, using washed dried beef as substrate, on the gastric juice of a small number of patients and, as it soon became evident that no significant difference could be detected by this method between the gastric juices from patients with pernicious anaemia and with other diseases, it was decided that no good purpose could be served by continuing the series. The *p*H-activity curves for the proteolysis of beef myoglobulin by the juices from normal subjects and cases of pernicious anaemia were also determined.

Experimental

Substrate. Dried beef was made by washing minced beef twice with dilute acetic acid at *p*H 5, filtering off the tissue, washing twice with 90% alcohol, then with absolute alcohol and finally with ether. The beef was dried in an incubator and powdered by passing through a mill.

The test was carried out as follows: a volume of the filtered gastric juice was depepsinized by treatment with sufficient of a solution containing 7·5% caseinogen and 4% Na_2CO_3 to bring the *p*H to 5·5, brought to a convenient volume and filtered.

In each of four stoppered tubes were placed 5 ml. of *p*H 5·5 acetate buffer solution and 2 drops of chloroform; into the first and second tubes were put 2 ml. of unheated depepsinized gastric juice and 20 mg. beef powder; into the third tube 2 ml. of depepsinized juice and into the fourth 2 ml. of heated (at 100° for 10 min.) depepsinized juice and 20 mg. beef powder. The contents of the first tube were deproteinized at once by the addition of 7 ml. of 15% trichloroacetic acid and those of the other tubes after incubation for 24 hr. Next day, the protein precipitate was filtered off through a small Whatman No. 42 paper and the non-protein nitrogen in a portion of the filtrate determined by the micro-Kjeldahl method. It was usually found that the amounts of non-protein nitrogen in the first, third and fourth tubes were equal, showing that no autolysis occurred in the gastric juice when incubated alone and that the heating was sufficient to inactivate any enzyme present. The amount of proteolysis is then given by the increase in non-protein nitrogen in the second tube when compared with the other three.

The results, calculated as mg. nitrogen produced by 1 ml. of juice, obtained in thirteen cases of pernicious anaemia are given in Table I and those from

Table I. *Cases of pernicious anaemia (achlorhydria before and after histamine stimulation)*

Case	mg. non-protein nitrogen produced per ml. juice	
	Before stimulus	After stimulus
1	0·82	—
2	1·21	4·82
3	1·24	—
4	4·18	1·32
5	0·50	—
6	2·44	2·84
7	0·56	0·02
8	0·00	1·62
9	0·62	1·66
10	0·90	—
11	4·52	3·32
12	Combined juice 0·16	
13	Combined juice 0·36	
Mean	1·65 ± 0·32	

eighteen cases of other diseases in Table II. In most of the cases the test was done on gastric juice obtained before and after stimulation by histamine injection or by gruel. The means with their standard errors for the three groups pernicious anaemia, other anaemias and cases other than anaemia are 1·65 ± 0·32, 1·16 ± 0·17 and 1·36 ± 0·13 respectively and do not differ significantly. The ranges covered by the results from the three groups are 0·00–4·82, 0·00–2·48

Table II. *Cases other than pernicious anaemia*

Case	Diagnosis	Free acid (units)		Hydrolysis: mg. N produced per ml. juice	
		In fasting contents	After stimulus gruel G or histamine H	Fasting contents	After stimulus
	Anaemias other than pernicious anaemia				
14	Anaemia of pregnancy	0	16 G	1·31	0·40
18	Hypochromic microcytic	0	0 G	2·48	1·14
21	,, ,,	0	0 G	—	1·30
22	,, ,,	3	33 G	0·68	1·78
23	,, ,,	0	0 G	—	0·00
24	,, ,,	0	0 H	0·52	0·78
25	Aplastic anaemia	11	26 H	2·22	1·46
31	Secondary to cirrhosis	24	45 G	0·54	—
17	Leukaemia with anaemia	0	0 H	—	0·88
20	,, ,,	25	80 H	2·04	1·07
Mean				1·16 ± 0·17	
	Non-anaemic patients				
15	Hyperpiesis	0	33 G	—	1·75
16	Rosacea, flatulent dyspepsia	26	40 G	1·40	1·30
19	Polycythaemia	50	70 H	0·76	1·47
26	Gastric ulcer	21	51 H	1·00	0·66
27	Cirrhosis	0	33 H	1·46	—
28	Thyrotoxicosis	0	0 H	1·92	—
29	Duodenal ulcer	35	38 H	1·25	—
30	Ileitis	35	31 H	2·00	—
Mean				1·36 ± 0·13	

and 0·66–2·00 respectively. The mean value obtained by Lasch for his series of cases of disease other than anaemia was $1·03 \pm 0·07$ with a range of 0·45–2·7. This differs little from the mean for our group of similar cases. Of his cases of pernicious anaemia, however, twelve out of seventeen gave zero values and five ranged from 0·08 to 0·18. The mean value for his cases of anaemia other than pernicious anaemia was $1·23 \pm 0·21$ with a range of 0·81–2·73, which also agrees well with our results. We can only conclude that Lasch's test as a diagnostic procedure is unreliable in our hands. The high results obtained for certain of our cases of pernicious anaemia, compared with those for the non-anaemic patients, have to be explained, since it is generally considered that the enzyme content of the gastric juice is diminished in the former. It must be remembered, however, that the juice is also diminished markedly in quantity [Wilkinson, 1932], so that what little peptic or other enzymic activity is present might be more concentrated.

The pH activity curves of gastric juices from normal subjects and cases of pernicious anaemia

The *p*H activity curves of gastric juice using standard buffered solutions of beef myoglobulin were determined by the method of Jones *et al.* [1938]. The buffered solutions covered a range of *p*H from 1·45 to 10·8. The results are shown in Fig. 1, in which the circles refer to the juice obtained from a case of pernicious anaemia (Case 13 in Table I), identical figures being obtained for the juice withdrawn before and after histamine stimulation. The triangles refer to combined samples of normal gastric juice. The substrate concentration was 0·56 mg. protein-nitrogen per ml. The ordinates are mg. non-protein-nitrogen produced by 1 ml. gastric juice in 1 hr. and are arranged so that the activity at *p*H 1·5 is the same for both kinds of gastric juice. It is evident that the

curve is the same for both types of juice and it is very similar to the curves obtained by Jones *et al.* [1938] for the action of pepsin and fractions of hog's stomach on beef myoglobulin and considered tentatively by them as solely due to the pepsin contained in the former two. Determinations were carried out at seven *p*H values between *p*H 5·8 and 10·8, but the activity at these reactions

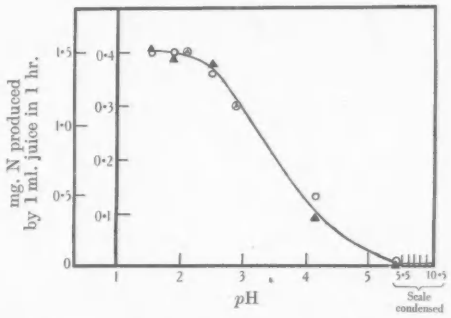

Fig. 1. Activity of gastric juice at different reactions: (1) activity of normal gastric juice (triangles); (2) activity of juice of pernicious anaemia (circles).

of the juice from cases of pernicious anaemia and from normal subjects was zero within experimental error (0·01 mg. N per ml.). The proteolysis obtained by us and other workers at *p*H 6 and 7·4 is probably the result of prolonged digestion, in spite of the great diminution in activity at these reactions.

Summary

1. The method suggested by Lasch [1937] for the determination of the intrinsic factor in gastric juice has been found to be unreliable.

2. The activities at reactions of *p*H 1·5–10·8 of the gastric juices from normal subjects and from patients with pernicious anaemia have been compared and found to follow an identical curve.

We are indebted to the Medical Research Council for grants with the aid of which this work was carried out.

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CLXXX. FURTHER OBSERVATIONS ON THE SYSTEM ASCORBIC ACID-GLUTATHIONE-ASCORBIC ACID-OXIDASE

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(*Received 2 June 1938*)

In a paper by Hopkins & Morgan [1936] it was shown that in aqueous solutions containing Szent Györgyi's oxidase together with ascorbic acid and glutathione, the former is protected by the latter from oxidation by the enzyme, while the glutathione is itself oxidized vicariously at a rate corresponding to that at which the ascorbic acid is oxidized when alone with the enzyme in similar concentrations. It was also shown that under the influence of similar enzyme preparations the reduction of dehydroascorbic acid by GSH is much faster than the oxidation of ascorbic acid, and also much faster than the reduction when uncatalysed.

Kertesz has since stated [1938] that he was unable to reproduce these experimental results. He found that GSH added to solutions of ascorbic acid containing the oxidase had no effect on the oxidation of the ascorbic acid at *p*H 6. At *p*H 7·4, while the GSH inhibited to some extent the oxidation of ascorbic acid, both substances were oxidized simultaneously. He found further that the reduction of dehydroascorbic acid by GSH was not catalysed by the enzyme preparation used by him.

It became clearly desirable that the experiments should be repeated, and the present paper deals with such repetitions together with some extensions. They have involved the use of many different enzyme preparations, and they show that the results published by Hopkins and Morgan are invariably reproducible. The enzyme preparations used by these authors were all derived from cabbages or from cauliflower florets. Kertesz employed cauliflowers, but also used cucumber juice. With no preparation did he obtain results comparable with those of the earlier workers. In the following sections experiments with cauliflower florets will be first described; certain others obtained with cucumber juice will afterwards receive special reference.

Unless an explanation which we will later venture to put forward is justified, we seem to be faced with an inexplicable difference in experimental experience.

Experimental

The cauliflowers employed have been both English-grown and imported. The concentration (or activity) of the oxidase has varied widely in different cases. In general the fresher the source of the supply the greater was the activity of the juice, but it varied apparently with the degree of maturity of the florets. The juice was expressed with avoidance of contact with Fe or Cu, and glass-distilled water was always used for making up solutions. The juice in each case was centrifuged before use, but was not otherwise fractionated. Owing to the wide variations in enzyme concentration we found it desirable to make a preliminary estimation of the rate at which each preparation oxidized a known

amount of ascorbic acid before proceeding to other experiments. The GSH was prepared in the laboratory; crystalline fractions almost free from any tendency to autoxidation being always employed. The ascorbic acid was usually that supplied by the British Drug Houses Ltd., but we have also used a standardized natural product kindly supplied by Sir Henry Dale. Both reactants were more stable than were apparently those employed by Kertesz. The methods of estimation were exactly those used by Hopkins & Morgan and also by Kertesz. A minor difference in procedure was that while our solutions were shaken continuously on a machine, in Kertesz's experiments they were shaken every 2 min. by hand. Our supply of GSSG was made by enzymic oxidation arrested before irreversible products were formed.

Experiments with cauliflower juice

In the work of Hopkins and Morgan, the behaviour of the system—ascorbic acid-GSH-oxidase—was chiefly studied at *p*H 7·4. It seemed desirable to determine through what range of variation in *p*H the protection of ascorbic acid and the other results obtained by them (if confirmed) would remain constant. Experiments were therefore made at *p*H 4·5, 6·0, 6·75, 7·4 and 8·25 respectively. The results are shown in Figs. 1–5.

Fig. 1 shows the relations displayed during aeration at *p*H 7·4. The enzyme preparation used (from a home-grown cauliflower) was initially very active, 50 mg. ascorbic acid (or, in the system an equivalent amount of GSH) being oxidized in <20 min. It was found that the oxidation of the ascorbic acid began just when a nitroprusside test first showed the disappearance of GSH. In order however that the relations could be more clearly shown (Fig. 1), the juice was diluted before use with twice its volume of glass-distilled water to reduce the velocity of change. The results reproduced exactly those of the earlier studies.

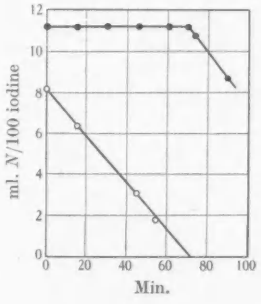

Fig. 1.

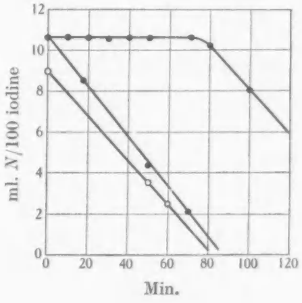

Fig. 2.

Fig. 1. Behaviour of system at *p*H 7·4. Upper line shows protection and subsequent oxidation of ascorbic acid. Lower line, course of oxidation of GSH in system. 1·75 mol. of former to 1 mol. of latter. McIlvaine's buffer (phosphate-citrate).

Fig. 2. System at *p*H 8·25. 2 mol. GSH to 1 mol. AA. Upper line, protection and subsequent oxidation of ascorbic acid. Middle line, oxidation of GSH in system. Lowest line, oxidation of ascorbic acid with enzyme in absence of GSH. McIlvaine's buffer.

The results at *p*H 8·25 are shown in Fig. 2. They were given by an undiluted preparation and are essentially the same as those obtained by Hopkins & Morgan, and by ourselves, at *p*H 7·4.

Fig. 3 shows the behaviour of the system at *p*H 6·75 and Fig. 4 that at *p*H 6. Both are again identical in essentials with the above.

Very different is the behaviour at *p*H 4·5 (Fig. 5). At this more acid reaction the system is dislocated; the protection of the ascorbic acid is imperfect and (as in Kertesz's experiments) both it and GSH are oxidized together.

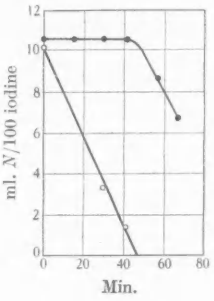

Fig. 3.

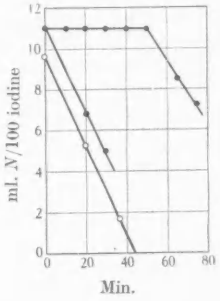

Fig. 4.

Fig. 3. System at *p*H 6·75. Details as in Fig. 1. GSH/ascorbic = 2 mol./1 mol.

Fig. 4. System at *p*H 6. Details as in Fig. 3.

It is easy to show from *p*H-velocity curves that this is because somewhere near *p*H 5 there is a critical point at which the velocity of the reducing process falls below that of the oxidative one.

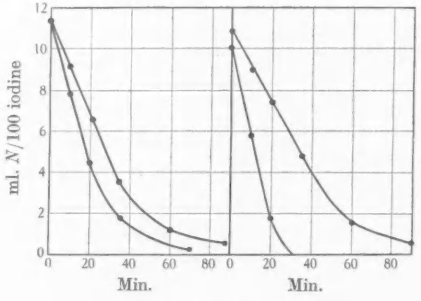

Fig. 5.

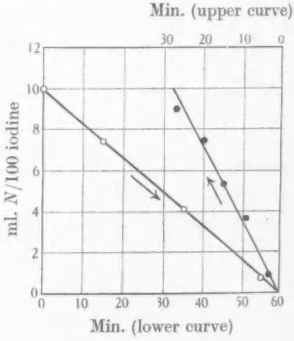

Fig. 6.

Fig. 5. System at *p*H 4·5. Two different enzyme preparations. Upper curve in each case, oxidation of GSH. Lower curves that of ascorbic acid. Ratio GSH/ascorbic = 2 mol./1 mol. McIlvaine's buffer.

Fig. 6. Lower line, enzymic oxidation of ascorbic acid; upper line, catalysed reduction of dehydroascorbic acid by GSH. For details see text. Sørensen's or McIlvaine's buffer.

Before the behaviour of the system at these low *p*H values can be understood, the behaviour of the mechanism which catalyses the reduction of ascorbic acid by GSH must receive reference. Its existence was clearly shown in the experiments described by Hopkins & Morgan, but Kertesz states that he was unable to repeat them. Pfankuch [1934] found that in potato juice the reduction of

dehydroascorbic acid by cysteine required enzymic catalysis for its occurrence as it did not occur in the previously heated juice. The influence of such a catalyst was first clearly shown in the experiments just mentioned.

Fig. 6 shows the relative velocities of the oxidation of ascorbic acid and its reduction by GSH as induced by a preparation of cauliflower juice. 88 mg. natural ascorbic acid in 100 ml. Sørensen's phosphate buffer at *p*H 6, together with 10 ml. enzyme preparation, were shaken in an open flask. The course of oxidation was followed by titrating successive 10 ml. samples. Oxidation was stopped at a point fixed by extending the strictly linear course to the base line when it could be assumed that reversible oxidation was complete. Samples of 10 ml. each were then transferred to a series of vacuum tubes in the bent-over hollow stoppers of which were 30·7 mg. GSH in 2 ml. buffer. After evacuation the GSH was mixed with the solutions and the tubes allowed to stand at room temperature (21°). The contents of the individual tubes were then titrated at successive time intervals. Oxidation and reduction thus progressed under the enzyme as originally added, but the volume of solution during reduction was greater than during oxidation in the proportion of 12 : 10. It should be noted that in this experiment only 2 mol. GSH were added for each mol. ascorbic acid; yet the reduction rate was linear until the process was almost complete, just as in the experiments which demonstrate that protection lasts till the GSH is practically all oxidized.

The uncatalysed reduction of ascorbic acid by GSH is a phenomenon long familiar in this laboratory. That it is much slower than the catalysed reaction was shown by an experiment—one of many with similar bearings—of which the results are given in Fig. 7. In this case the GSSG was made by oxidizing GSH with iodine. The I_2 was quantitatively removed by lead acetate and a minute excess of Pb by K_2SO_4. Reduction was carried out in vacuum tubes; 4 tubes in each of 3 series. One series contained the enzyme solution, in a second this was replaced by an equal volume of water, in the third each tube contained 0·022 mg. Cu. Otherwise the content of every tube was the same, 3 mol. GSH being present for each mol. ascorbic acid. The *p*H was 6. The evacuated tubes stood at room temperature (22°) and their contents were titrated at successive intervals. In Fig. 7 the upper curve shows the rate of the enzyme-catalysed reduction, the lower curves that of reduction in the absence of enzyme. Of the latter two, which are nearly identical, the lower shows that Cu does not catalyse the reduction process. With enzyme the dehydroascorbic acid was completely reduced in 15 min. or less, without it only 40% was reduced in 1 hr. It is the experience of this laboratory that similar results are always to be obtained with the two varieties of *Brassica* that have so far been employed. In his paper Kertesz [1938] remarks "The results obtained with the system shown in their Fig. 3 (i.e. complete protection of the ascorbic acid so long as GSH is present) were observed at *p*H 7·4. It is doubtful whether the experiment could have been repeated at *p*H 6, although Hopkins & Morgan make no statement to this effect." Fig. 6 of the present paper shows however that there is complete protection at that *p*H while the catalysis of reduction at *p*H 6 is fully confirmed.

It is important to note the general effect of *p*H on the relative activities of the oxidase and the reducing enzyme (or catalytic system).

Fig. 8 is based on determinations of the initial velocities of oxidation and reduction respectively at varying *p*H values. They were made with an active specimen of cauliflower juice. The flatter and unsymmetrical curve (*A*) relates to oxidation velocities, the symmetrical (*B*) to those of reduction. The points on the curves are from estimations of the amount of change in either direction

induced in 15 min. It is seen that through a range of high *p*H values reduction is a faster process than oxidation while through a range of lower values reduction is the slower. In the complete system protection of ascorbic acid during the presence of GSH would be expected throughout the former range and not within the latter. In a number of less complete studies we found that the transition point where the curves cross lay between *p*H 4·5 and 5. The exp. of Fig. 8 was

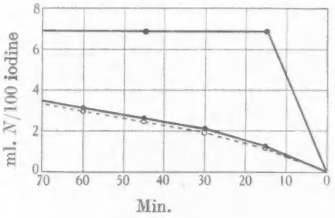

Fig. 7.

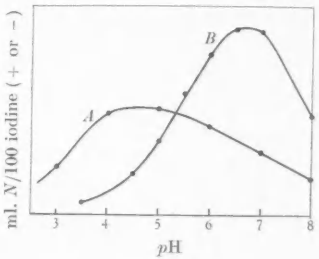

Fig. 8.

Fig. 7. Curves run from right to left. Upper, the catalysed reduction by GSH. Lower continuous line, the rate of reduction when uncatalysed. Lower broken line, rate of reduction in presence of copper. Sørensen's buffer.

Fig. 8. *p*H-velocity curves. Curve *A* gives the initial rates of oxidation measured at different *p*H values. Curve *B* applies similarly to rates of reduction in the presence of the enzyme of (enzymically prepared) dehydroascorbic acid. McIlvaine's buffer.

carried out for the construction of more complete curves for publication, and it happened that the transition point in this case was just above *p*H 5. It would indeed be satisfactory if all the aspects of behaviour displayed by the system could be studied with the same preparation and without delay. This we have found to be impossible. While it is true, as various authors have stated, that the oxidase itself is relatively stable, the reducing mechanism is much less so. Its activity may be lessened in preparations kept overnight, and falls off slowly when an expressed juice is shaken in the air in the absence of any added substrate.

From these considerations it will be understood that in observations made at a *p*H only a little above 5, the degree of protection may be uncertain. At *p*H 5·25 for instance we have obtained typical protection with a fresh juice, but none with the same juice after standing for 3 days, though even then there was sufficient of the reducing factors left for protection at *p*H 7·4.

Fig. 8 makes clear the general nature of the influence of *p*H variations on the relative rates of oxidation and reduction, and explains completely the disappearance of protection at *p*H 4·5 (Fig. 5).

Hopkins & Morgan [1936] reported a circumstance involving some difficulty in explanation. Though typical protection was obtained in the system when dialysed juice was employed, dialysis nevertheless diminished the power of preparations to promote reduction. Kertesz [1938] in commenting on this remarks, "The observation that dialysed cauliflower juice could produce complete protection of ascorbic acid in the presence of glutathione but could not catalyse the reduction of dehydroascorbic acid by glutathione makes the explanation given for the mechanism somewhat uncertain." This does not state the facts correctly. With dialysed juice the initial velocity of reduction was as

great as with undialysed, but the rate fell off before reduction was complete. This circumstance still lacks explanation, but the additional evidence given in this paper for the relative instability of the reducing factor (or factors) perhaps affords some help. It is possible that dialysis reduces the amount of some protective agent. As stated above, the reducing activity of a preparation falls off when it is shaken aerobically by itself. This destruction may proceed faster in the dialysed juice.

We venture to include here parenthetically the results of an experiment carried out by Hopkins & Morgan but not published, which shows how justly ascorbic acid might be looked upon as a coenzyme for the oxidation of GSH. Dialysed juice was employed so that all pre-existing ascorbic acid was removed and GSH therefore quite unaffected by the oxidase. In Fig. 9 is shown the effect of subsequently adding minute amounts of ascorbic acid on the rate of GSH oxidation.

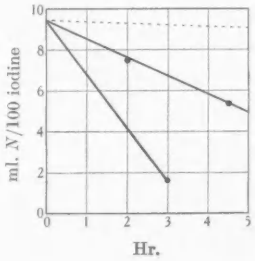

Fig. 9.

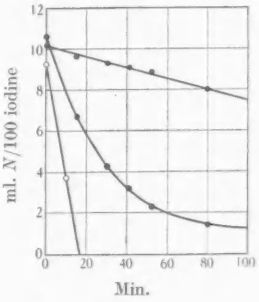

Fig. 10.

Fig. 9. Exp. with dialysed juice. Broken line, GSH with enzyme alone (unaffected). Middle line, oxidation of same in presence of 0·1 mg. ascorbic acid. Lower line with 1·0 mg. Sørensen's buffer, *p*H 7·4.

Fig. 10. System at *p*H 7·0 with cucumber juice. Upper line, oxidation of ascorbic acid in system. Middle curve, oxidation of GSH in system. Left-hand line, oxidation of ascorbic acid when alone with enzyme. 2 mol. GSH to 1 mol. ascorbic acid. McIlvaine's buffer.

Experiments with cucumber juice

Until the appearance of Kertesz's paper we had had no experience of the use of preparations from the cucumber, which were used in the greater number of his experiments. It is well known that enzyme systems differ widely in plants of different families, and they may well differ in different tissues of the same plant. One thing is sure, however. Unlike some other fruits, e.g. the apple [Johnson & Zilva, 1937], the cucumber contains the ascorbic acid oxidase [Kertesz, 1938]. It also contains the reducing system, but in all the specimens we have employed the concentration of this relative to that of the oxidase is small when compared with their mutual relations in the leaves and florets of the *Brassica* plants. In experiments such as those on which Fig. 8 was based, we have found that the velocity of reduction never rises nearly so far above that of oxidation as is shown in this figure, and it remains above it during a much smaller range of *p*H values. The maximum velocity of reduction is at a somewhat less acid reaction (about *p*H 7) than that of oxidation. It further seems sure that for some reason the reducing system is less stable or less protected in

cucumber juice than in that of cauliflower or cabbage. This indeed seems to be the case with the oxidase also. When the two juices without any addition are thoroughly aerated side by side by shaking at *p*H 6, the resultant falling off of oxidase activity is much more marked in the cucumber juice than in the other. It may be however that a lower relative concentration of the reducing system in cucumber constitutes the main difference between them.

The results we have obtained with cucumbers do not differ fundamentally from those published by Kertesz, except that at *p*H values near to 7 we have found greater protection by GSH than he reported. At *p*H 6 it may be small or absent. Fig. 10 gives results typical of those we have obtained at *p*H 7·0. The departure of the GSH oxidation from the linear progress which was always found in the experiments with cauliflower juice may have been due here to a gradual destruction of the less stable reducing system.

Discussion

In this paper it is shown that all the results obtained by Hopkins & Morgan in their study of the system under reference are reproducible, and we may add that when the experimental conditions are properly defined the reproduction has been in our experience invariable. In so far as he used the same materials as those used by them, the inability of Kertesz to obtain the same results is exceedingly difficult to explain.

The greater number of Kertesz's experiments were however carried out with preparations from cucumbers, and with this material our own results, though not identical with his, come closer to them. It is so well known that the enzymes in plants of different orders or families vary in nature and organization, and so probable that those in different tissues even of the same plant may differ, that it was not justifiable for this author to apply results obtained from material different from that used by Hopkins & Morgan in criticism of their findings. This he does, at least by implication.

It is true that he also used cauliflowers, but apparently in a few experiments only. There remains nevertheless the difficulty of reconciling his results with those of Hopkins & Morgan and our own. It is perhaps possible though very improbable that the explanation might be found in the different provenance of the plants employed; Kertesz's experiments being done in Stockholm. This becomes the less likely since in the Cambridge experiments plants imported from the Continent and others grown on different soils in England have been employed. A more likely explanation is to be found in the instability of the reducing mechanism, of which Kertesz was not aware. He has kindly informed us that the plants used had probably travelled a long distance before they were purchased, and that the expressed juices were not always used immediately after their preparation.

We are particularly concerned to emphasize the quite certain presence in the plants which, because of the nature of Szent Györgyi's pioneer studies, were used by ourselves, of an agent which actively catalyses the reduction of ascorbic acid by glutathione. Its distribution has yet to be explored, but even if this proves to be limited, it is yet an enzyme (or enzyme in association) of which the kinetics must possess a special interest. In any study of these its relative instability must be remembered. An endeavour is being made in this laboratory to separate it from the associated oxidase.

SUMMARY

The experiments of Hopkins & Morgan [1936] on the system ascorbic acid-glutathione-ascorbic acid-oxidase, which Kertesz [1938] was apparently unable to confirm, have been repeated and extended, and the original results completely confirmed. In particular the effect of variations in *p*H on the enzymic activities of oxidase preparations has been determined.

The expressed juice from cucumbers differs in certain noteworthy respects from that obtained from cabbages and cauliflowers. It contains the oxidase, as Kertesz has shown, and it also contains a mechanism capable of catalysing the reduction of ascorbic acid by glutathione. The latter however is present in relatively low concentration.

Possible reasons for Kertesz's inability to repeat the earlier experiments are discussed.

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CLXXXI. ALCOHOL DEHYDROGENASE OF ANIMAL TISSUES

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It was first found by Batelli & Stern [1910] that extracts from various animal tissues are able to oxidize alcohol. More recent investigations and attempts to isolate the enzyme responsible for propyl alcohol oxidation were carried out by Reichel & Köhle [1935]. Alcohol oxidation in tissue slices from normal and fasting animals was studied by Leloir & Munoz [1938]. Bernheim [1938] investigated the influence of alloxan on the alcohol oxidation in tissue extracts.

The present work was undertaken in connexion with the study of the liver aldehyde mutase [Dixon & Lutwak-Mann, 1937]. This enzyme catalyses the dismutation of $CH_3.CHO$ to C_2H_5OH and $CH_3.COOH$ and it was of interest to investigate the alcohol dehydrogenase in the liver by methods similar to those employed for the mutase study.

Materials

Horse liver was used throughout as the source of the animal alcohol dehydrogenase. Livers from other animals (dog, pig, sheep) were also found to contain the enzyme in large quantities. A number of preparations were made in which acetone and ammonium sulphate were used for precipitation.

1. *Acetone preparation.* Minced horse liver is mixed with an equal volume of water and after standing a few hours at room temperature the large particles are removed by centrifuging. The aqueous extract is heated to 52° for 15 min. to remove inactive protein, then cooled and precipitated with 3 vol. acetone. The precipitate is centrifuged and resuspended by grinding with a small amount of water. The insoluble part is discarded and the extract is then precipitated with 2 vol. acetone. (Sometimes a 3 : 1 mixture of acetone and alcohol is used.) This procedure is repeated twice. The final acetone precipitate is dried *in vacuo* over $CaCl_2$. This preparation usually contained in addition to the alcohol dehydrogenase an active aldehyde mutase, but gave negative results for the aldehyde oxidase.

2. *Ammonium sulphate preparations.* (*a*) The aqueous liver extract is precipitated with 3 vol. saturated ammonium sulphate, the precipitate is filtered off and partially dried in air, then completely dried *in vacuo* over H_2SO_4 and KOH. (*b*) The liver extract is heated to 52° for a few minutes, the resulting precipitate discarded and the filtrate precipitated using 30 g. solid ammonium sulphate for every 100 ml. fluid. (*c*) The preparation obtained in (*a*) is taken up in a small quantity of water and dialysed against running tap water for 24 hr. The precipitate formed on dialysis is filtered off and the fluid precipitated with acetone in the same way as described in the acetone preparation.

A number of experiments were also carried out using acetic bacteria as the source of a system capable of alcohol oxidation. Cultures of *Acetobacter suboxydans* grown on glycerol-yeast water medium were spun off, washed twice with water and finally suspended in water. This suspension was used for experiments and was found to retain its activity for several days at 0°.

Coenzymes. Cozymase (70% pure) was prepared from baker's yeast by a modification and combination of Myrbäck's methods as described by Green & Brosteaux [1936]. Coenzyme II from red blood cells and flavoprotein from Lebedew juice were prepared by the methods of Warburg & Christian [1931; 1932]. Cocarboxylase and adenosinetriphosphate were obtained as described by Lohmann [1931] and Lohmann & Schuster [1937].

Methods

The enzymic oxidation of alcohol was tested mainly by two methods. (1) Anaerobically, in presence of methylene blue as hydrogen acceptor following the usual Thunberg technique. Tubes with hollow stoppers were used from which the substrate, methylene blue and other reactants were emptied into the tube containing the enzyme solution after the latter had been exhausted and filled twice with O_2-free N_2, and the reduction time of methylene blue at 37° was measured. (2) Aerobically, O_2 uptake was measured using Barcroft differential manometers with side bulbs [Keilin & Hartree, 1935]. The enzyme solution in *M*/30 phosphate buffer was placed in the right-hand flask only and the phosphate buffer in the other flask. The inner tubes contained KOH-soaked filter papers. Alcohol and other substances were emptied into the flasks from both side bulbs after 15 min. equilibration at 37°.

Properties of the alcohol dehydrogenase system

Coenzymes. The yeast alcohol dehydrogenase has been shown to require for its activity the presence of cozymase (diphosphopyridine nucleotide, coenzyme I) and of flavoprotein (yellow enzyme). In order to determine whether these two substances play a similar role in the oxidation of alcohol by preparations from animal tissues it was essential to have a coenzyme-free enzyme preparation.

Ordinary horse liver acetone preparations were unsuitable for this purpose since they always contain large amounts of cozymase which cannot be removed even by prolonged dialysis [Reichel & Köhle, 1935; Dixon & Lutwak-Mann, 1937]. The last-named authors used dog liver acetone preparations which contain practically no cozymase owing to its quick decomposition by this particular liver tissue. A very convenient and simple method has now been found to obtain horse liver preparations completely free from cozymase. If the liver extract be first treated with ammonium sulphate (as under (*a*), p. 1364) and either used directly or precipitated with acetone after removal of ammonium sulphate (as under (*c*), p. 1364) it then appears to be cozymase-free. On testing these preparations for aldehyde mutase, unless cozymase was added they showed no trace of activity. It can also be seen from Table I, Exps. 1–6, that the alcohol dehydrogenase prepared in this way catalyses the reaction only on addition of cozymase. Coenzyme II (triphosphopyridine nucleotide), cocarboxylase, adenosinetriphosphate, glutathione, known to act as coenzymes in various enzymic

Table I. *Effect of cozymase and flavoprotein on various liver alcohol dehydrogenase preparations*

Thunberg tubes with 20 mg. enzyme preparation, 1 mg. methylene blue, 2 ml. phosphate buffer *M*/30, *p*H = 7·6.

Ammonium sulphate preparation	ml. alcohol 96%	mg. cozymase	mg. flavoprotein	Reduction time min.
Exp. 1	0·1	—	—	∞
2	0·1	0·02	—	(Several hr.)
3	0·1	0·10	—	35
4	0·1	0·40	—	7
5	0·1	1·00	—	6
6	0·1	2·00	—	6
7	0·1	0·50	2·0	7
8	—	0·50	2·0	∞
Acetone preparation				
9	0·1	—	—	60
10	0·1	0·30	—	20
11	0·1	0·60	—	20
12	0·1	0·60	2·0	18

systems, and also nicotinamide, were found to be completely inactive. Recently Quibell [1938] has also found cozymase to be the specific coenzyme of the animal alcohol dehydrogenase.

That cozymase is also involved in the oxidation of alcohol by the acetic bacteria was shown in the following way. Fresh untreated suspensions of *Acetobacter suboxydans* very vigorously oxidize alcohol, both aerobically and anaerobically, and it is difficult to produce an acceleration of the reaction by addition of cozymase. When, however, the suspension was saturated with ammonium sulphate and after a few hours exhaustively dialysed, a marked increase in the velocity of the reaction was found on addition of cozymase.

Table II. *Effect of cozymase on oxygen uptake in presence of alcohol by acetic bacteria*

In Barcroft manometers, bacterial suspension in phosphate buffer $M/30$, pH $=6{\cdot}5$, 2 mg. alcohol, 0·3 ml. 20% KOH in inner tube, total volume 3·3 ml.

	O_2 uptake μl./20 min.
Fresh suspension	320
Do. +0·4 mg. cozymase	350
Treated with ammonium sulphate and dialysed	90
Do. +0·4 mg. cozymase	200

With regard to flavoprotein it can be seen from Table I, Exps. 7, 12, that it has only very little effect on the reaction. Dewan & Green [1938] were also unable to see any marked influence of flavoprotein in animal enzyme systems.

Coenzyme-factor. Stability of the alcohol dehydrogenase

The belief that the alcohol dehydrogenase from animal tissues is a very labile and unstable enzyme [Batelli & Stern, 1910; Reichel & Köhle, 1935] is disproved by the following observations. Two types of liver alcohol dehydrogenase preparations, (1) the usual acetone preparation, and (2) an ammonium sulphate preparation, made by method (*a*) above, were tested by the methylene blue technique every 2 weeks for a period of 4 months. The crude ammonium sulphate preparation seemed to be the more active one and its activity remained practically unchanged during the long period of observation. The acetone preparation gave lower initial values and seemed to deteriorat egradually on standing. This suggested the possibility of a special agent necessary for alcohol dehydrogenase activity, present apparently in the cruder ammonium sulphate preparation but partly destroyed in the acetone-treated preparation, in which the agent also seemed to undergo progressive decomposition on storing.

At this stage a note was published by Dewan & Green [1937] on the necessity of a special oxidation catalyst which they called the "coenzyme factor" in all enzyme systems where cozymase was involved. I had the opportunity of testing the influence of the coenzyme factor prepared from heart muscle and kindly given to me by Drs Dewan & Green. The experiment has been described in their paper [1938, p. 633]; it shows a very pronounced acceleration of the reaction on the addition of the factor preparation from heart muscle to an acetone liver preparation of alcohol dehydrogenase.

In order to prove that the differences described above in the behaviour of the crude ammonium sulphate and the acetone preparations were due solely to their unequal contents of the coenzyme factor, the presence of the factor in the liver had to be shown. Liver mince was washed with tap water for 24 hr. until

Table III. *Stability of the liver alcohol preparations on storing and effect of the coenzyme factor*

Thunberg tubes with 40 mg. enzyme preparation, 0·5 mg. cozymase, 1 mg. methylene blue, 0·1 ml. 96% alcohol, 2 ml. phosphate buffer *p*H = 7·6. 1 ml. coenzyme factor preparation (sheep liver).

Age of alcohol dehydrogenase preparation		Reduction time, min. Ammonium sulphate	Acetone
Fresh preparation	Without factor	4 min.	58 min.
	With factor	4 min. 50 sec.	4 min. 30 sec.
2 weeks old	Without factor	4 min.	60 min.
	With factor	4 min. 50 sec.	4 min. 30 sec.
2 months old	Without factor	5 min. 30 sec.	3 hr.
	With factor	4 min.	4 min.
4 months old	Without factor	6 min.	Several hr.
	With factor	4 min.	6 min.

almost colourless, ground in water with sand, the coarse particles were removed by centrifuging or straining through muslin and the resulting uniform suspension was used. Alone it gave negative results when tested for alcohol dehydrogenase, aldehyde mutase and aldehyde dehydrogenase, but added to the acetone preparation of alcohol dehydrogenase it produced a very marked acceleration of the methylene blue reduction (Table III). Washed liver suspensions as used in these experiments retain their activity for several days when kept at 0°.

The coenzyme factor has been shown to be acetone-sensitive and thermolabile [Dewan & Green, 1937] but resistant to ammonium sulphate treatment [Euler & Hellström, 1938]. This explains the poor activity of the acetone preparations from the beginning and also their apparent continual deterioration on standing. From Table III it can be seen that when supplemented by the coenzyme factor there is no change in the activity of the acetone preparation during the whole period (4 months) of the experiment. In the ammonium sulphate preparation no acceleration was seen on addition of the factor. It is however enough to heat the liver extract for a short time to 52° before the ammonium sulphate precipitation (as described under (*b*) above), to obtain a preparation giving results similar to those of the acetone preparations.

Effects similar to those with the washed liver suspensions were also obtained with preparations of coenzyme factor made from skeletal muscle, spleen and brain tissue. Dewan & Green [1938] and Euler & Haasse [1938] state that the factor is present in a great variety of animal tissues.

In addition to the above results which seem to disprove completely the alleged instability of the alcohol dehydrogenase, I was never able to confirm statements regarding the sensitivity of the enzyme towards O_2 or on standing in solution. There was no significant loss of activity on aeration at 37° or on prolonged dialysis or in the solution after standing for 3–4 days.

Optimum p*H*. Reichel & Köhle [1935] state that the *p*H optimum of their preparations of liver alcohol dehydrogenase is 6·7–7·3. Using the methylene blue technique the optimum was found to be at *p*H 7·6, with a rapid fall below *p*H 7. The shift towards the alkaline side may be caused by the addition in these experiments of the coenzyme factor which is very acid-sensitive and comparatively resistant to alkali.

When however the bacterial alcohol oxidase was tested at various reactions from *p*H 4·5 to 8·3, the optimum was found at *p*H 5.

Estimation of the reaction product formed anaerobically

Data for anaerobic experiments on the alcohol dehydrogenase are given in Tables I and III. It remained however to estimate the product of the reaction with and without coenzyme factor.

In order to obtain enough material for a chemical determination samples 10 times larger than those normally required for ordinary Thunberg experiments were put up. Large (70 ml.) Thunberg tubes were found most convenient; their hollow stoppers were filled with *M* phosphoric acid to inactivate the enzyme at the end of the reaction, which was indicated by the reduction of the methylene blue. After efficient cooling in ice-water the fluid was quantitatively transferred to the distillation flask of the Friedemann *et al.* [1927] apparatus. The distillation into $NaHSO_3$ was continued for 15 min. and the estimation of acetaldehyde was carried out iodimetrically.

The actual composition of the various samples and controls together with the results obtained are given in Table IV.

Table IV. *Chemical estimations of acetaldehyde*

In Thunberg tubes (70 ml.) 5 ml. dialysed enzyme solution (=0·3 g. acetone preparation), 4 ml. phosphate buffer *M*/20, *p*H 7·6, 10 mg. methylene blue (MB), 5 mg. cozymase, 0·3 ml. 96% alcohol, 4 ml. factor preparation. In hollow stoppers 10 ml. *M* phosphoric acid.

In control experiments acid was added to the enzyme and factor before the other reagents.

1 ml. *M*/100 I_2=0·22 mg. acetaldehyde.

1 mol. MB (355) corresponds to 1 mol. acetaldehyde (44).

	Reduction time min.	ml. *M*/100 I_2
Controls		
1. Enzyme + factor + cozymase + alcohol + MB	—	1·95
2. Factor (sheep liver, heart muscle)	—	0·10
3. Enzyme + factor (liver) + cozymase + MB + 1 mg. acetaldehyde	—	6·80
Experiments		
1. Enzyme + cozymase + alcohol + MB	80	4·45
2. Enzyme + cozymase + alcohol + heart muscle factor + MB	12	6·00
3. Enzyme + cozymase + alcohol + skeletal muscle factor + MB	16	6·60
4. Enzyme + cozymase + alcohol + liver factor + MB	8	2·20
5. Enzyme + cozymase + alcohol + liver factor + MB	10	2·35
6. Enzyme + cozymase + 1 mg. aldehyde + liver factor + MB, incubated for 10 min. at 37°	—	6·60

It can be seen from Table IV that acetaldehyde is produced in samples without additional coenzyme factor and also when a washed heart muscle suspension is added as the source of the factor. Acetaldehyde dinitrophenylhydrazone was also obtained in similar experiments by precipitation with 2:4-dinitrophenylhydrazine after removal of the proteins with trichloroacetic acid and adsorption of methylene blue on kaolin.

In all cases however where washed liver suspensions provided the coenzyme factor, acetaldehyde formation could not be detected. A control experiment (Table IV, Exp. 6) where, instead of alcohol, a known amount of acetaldehyde was incubated for 8 min. with the experimental mixture and recovered quantitatively, excluded the possibility of acetaldehyde disappearance due to dismutation or oxidation. No satisfactory explanation of this phenomenon could be found and it is not easy to see what could be the reaction product of alcohol dehydrogenation in this case.

Aerobic experiments on the alcohol dehydrogenase

Several preliminary experiments were first carried out on 1 : 1 aqueous liver extracts in which O_2 uptake was measured on addition of alcohol and cozymase. A very rapid and marked increase in O_2 uptake by the extracts was observed with alcohol and similarly, but to a smaller extent, with glycerol. It should however be pointed out that there was always a large "blank" O_2 uptake in the extracts which did not disappear even on very long autolysis and dialysis. The observed increase in O_2 uptake on addition of alcohol very likely represented some complicated reaction between alcohol (or its oxidation product) and the substrates already present in the extract.

When however the O_2 uptakes of the acetone or ammonium sulphate preparations of liver alcohol dehydrogenase were tested manometrically on addition of alcohol and cozymase, it was found that practically no O_2 was taken up unless a carrier was provided. Methylene blue was found to be a suitable carrier, pyocyanine acted equally well (Table V, Exps. 2–4), quinone in small concentrations was useless, in larger concentrations it inhibited the enzyme. Even in the presence of methylene blue the reaction still remained very slow. Some acceleration was evident when $M/6$ semicarbazide was added as acetaldehyde fixative (Table V, Exp. 7). Addition of suspensions of coenzyme factor from liver or skeletal muscle had no effect on the O_2 uptake; when however a washed heart muscle suspension was used a definite acceleration of the reaction was obtained (Table V, Exps. 8, 10). The explanation probably lies in the fact that when tested for O_2 uptake in presence of *p*-phenylenediamine, the heart muscle suspension showed a considerable activity due to cytochrome oxidase even without addition of cytochrome *c*, whereas the liver and skeletal muscle suspensions yielded negative results.

Table V. *O_2 uptake of the liver alcohol dehydrogenase preparation*

In Barcroft manometers. 30 mg. enzyme preparation, 5 mg. alcohol, 0·5 mg. cozymase, 0·1 and 1 mg. methylene blue (MB), 1 mg. pyocyanine hydrochloride, 2 mg. flavoprotein, 0·5 ml. $M/2$ semicarbazide, 1 ml. washed heart muscle or skeletal muscle preparation. Phosphate buffer $M/30$, *p*H 7·4, total volume 3·3 ml.

Enzyme + cozymase + alcohol with the addition of:	O_2 uptake μl./80 min.
1. —	20
2. 0·1 mg. MB	50
3. 1·0 mg. MB	120
4. 1·0 mg. pyocyanine	110
5. 2·0 mg. flavoprotein	30
6. 2·0 mg. flavoprotein + 1·0 mg. MB	90
7. $M/2$ semicarbazide	170
8. Washed heart muscle suspension	240
9. Washed skeletal muscle suspension	40
10. Washed heart muscle suspension	200

Chemical estimations of the reaction product in aerobic experiments were made, but gave unsatisfactory results owing mainly to the very prolonged reaction time (3–4 hr.) before theoretical values were reached. The presence of volatile acid (acetic acid) in these experiments especially with some of the more crude preparations explained the small yields of acetaldehyde. It pointed to the fact that acetaldehyde partly underwent either oxidation or dismutation to acetic acid.

Various alcohols. The activity of the liver alcohol dehydrogenase was tested in presence of other alcohols, mainly by the Thunberg technique. Propyl

alcohol gave values similar to ethyl alcohol; with methyl and amyl alcohols positive results were obtained. With saligenin, glycerol and α-glycerophosphate negative results were obtained even on addition of the coenzyme factor.

Table VI. *Various alcohols*

Thunberg tubes with 20 mg. enzyme preparation, 30 mg. substrate, 0·5 mg. cozymase, 1 mg. methylene blue, 1 ml. coenzyme factor, 2 ml. phosphate buffer *p*H 7·6.

	Reduction time
1. Ethyl alcohol	1 min. 35 sec.
2. Propyl alcohol	2 min.
3. Amyl alcohol	5 min. 35 sec.
4. Methyl alcohol	11 min.
5. Saligenin	∞
6. Glycerol	∞
7. α-Glycerophosphate	∞

The comparatively slow oxidation of methyl alcohol (Table VI, Exp. 4) suggested the possibility of a poisoning of the enzyme by H.CHO as the reaction product. But it was found that H.CHO in small concentrations has little effect on the liver alcohol dehydrogenase and incidentally it was discovered that it undergoes in the liver preparations a typical dismutation to CH_3OH and H.COOH. Large quantities of H.CHO (and also of CH_3.CHO) produce a definite inhibition of the alcohol dehydrogenase which is partly reversible after removal of the H.CHO by dialysis.

The presence of methyl alcohol has no inhibitory effect on the oxidation of C_2H_5OH in liver enzyme preparations. In this respect the bacterial alcohol oxidase shows an interesting behaviour. It hardly oxidizes CH_3OH at all, but in the presence of CH_3OH the oxidation of C_2H_5OH is almost completely abolished. That this is due to a specific influence of the CH_3OH on the alcohol enzyme and not to a general poisoning effect on the bacteria was shown by the fact that other enzymes (aldehyde and/or glucose oxidation) of these acetic bacteria were found to be entirely unaffected by CH_3OH.

Inhibitors

Dixon [1937] has found that *M*/1000 iodoacetate completely inhibits the yeast alcohol dehydrogenase. However, negative results or only slight inhibition was obtained with *M*/100 iodoacetate on the animal alcohol dehydrogenase (Table VII; Table VIII, Exps. 3–6). It seemed desirable to examine more closely this difference in the behaviour of the yeast and liver enzymes.

It was thought that the negative results with the liver enzyme preparation might be due to the high content of combined and free SH-groups (glutathione) of the proteins and consequently to a complete decomposition of the iodoacetate during the incubation with the liver enzyme solution. To test this possibility the following procedure was chosen. The yeast alcohol dehydrogenase was prepared by the method used by Dixon [1937] and found to be completely inhibited by *M*/100 iodoacetate. Then varying amounts of the liver enzyme preparation, some of them after exhaustive dialysis and also in some cases after heat-inactivation, were incubated with iodoacetate (final concentration *M*/20) for 5–10 min. at 37°. Then they were mixed with the yeast enzyme and the mixture was again incubated for some time at 37°. The activity was then tested by means of the methylene blue technique in the usual way. It was found that the inhibitory effect of iodoacetate upon the yeast enzyme was almost completely abolished in samples where comparatively large quantities of the

Table VII. *Effect of iodoacetate on the liver alcohol dehydrogenase*

30 mg. enzyme preparation, 0·5 mg. cozymase, 0·1 mg. alcohol 96%, 1 mg. methylene blue, 1 ml. factor preparation, iodoacetate (IAA) to give $M/50$ or $M/100$ final concentration. The enzyme solution was incubated with iodoacetate for 15 min. at 37° before the actual experiment.

	Reduction time
A. Ammonium sulphate preparation	
1. No IAA	6 min.
2. +IAA $M/100$	7 min.
3. +IAA $M/50$	7 min. 50 sec.
B. Acetone preparation	
Not dialysed:	
1. No IAA (without "factor")	28 min.
2. +IAA $M/100$ (without "factor")	29 min.
Dialysed:	
3. No IAA (without "factor")	30 min.
4. +IAA $M/100$ (without "factor")	36 min.
5. No IAA (with "factor")	3 min. 10 sec.
6. +IAA $M/100$ (with "factor")	5 min. 50 sec.

liver preparation, boiled or unboiled, were incubated with iodoacetate (Table VIII, B, Exps. 1, 3). If, however, small amounts of the liver enzyme were used, or after a very prolonged dialysis, the decomposition of iodoacetate by the SH-groups of the liver enzyme was comparatively small and there was enough iodoacetate left to exert its usual poisoning effect upon the yeast alcohol dehydrogenase (Table VIII, B, Exps. 2, 4). The liver enzyme nevertheless remained uninhibited (Table VIII, A, Exps. 4, 6).

Table VIII. *Effect of iodoacetate in mixtures of yeast—and liver—alcohol dehydrogenase preparations*

Thunberg tubes. 3% enzyme solutions in phosphate buffer $M/50$, pH 7·4; 0·5 mg. cozymase, 1 ml. $M/2000$ MB.

	ml. yeast enzyme	ml. liver enzyme	Final concentration of IAA	Reduction time
A. 1.	1·00	—	—	3 min. 35 sec.
2.	1·00	—	$M/100$	∞
3.	—	0·50, not dialysed	—	18 min.
4.	—	0·50, not dialysed	$M/100$	20 min.
5.	—	0·50, dialysed	—	25 min.
6.	—	0·50, dialysed	$M/100$	33 min.
7.	1·00	0·50, not dialysed	—	3 min. 50 sec.
8.	1·00	0·10, not dialysed	—	3 min.

B. Samples of liver enzyme + IAA (final concentration $M/50$) were incubated for 10 min. at 37°. 1 ml. of yeast enzyme solution was added and the mixture again incubated for 10 min. at 37° before testing with MB.

Yeast enzyme added to:	Reduction time
1. 0·50 ml. liver enzyme (not dialysed) + IAA	5 min. 45 sec.
2. 0·10 ml. liver enzyme (not dialysed) + IAA	60 min.
3. 0·50 ml. liver enzyme (boiled, not dialysed) + IAA	7 min. 30 sec.
4. 0·50 ml. liver enzyme (dialysed) + IAA	50 min.

Yet another experiment was done to prove that the liver alcohol dehydrogenase is resistant to iodoacetate. In a liver preparation which contained both the alcohol dehydrogenase and the aldehyde mutase, $M/80$ iodoacetate inhibited the mutase almost completely and had only a very small effect on the dehydrogenase.

The bacterial alcohol oxidase tested aerobically gave similarly negative results after prolonged incubation with iodoacetate. In anaerobic experiments a slight but reproducible inhibition was recorded.

A few other substances known to act as inhibitors in various enzyme systems were tested and their actions both on the liver alcohol dehydrogenase and aldehyde mutase compared. Urethane, oxalate, maleate and pyrophosphate produce a fairly large inhibition of the alcohol dehydrogenase (30–50 %) but none of the mutase. Both enzymes are cyanide stable. Quinine, morphine and nicotine in low concentration act as inhibitors of the mutase but have no effect on the alcohol dehydrogenase.

In experiments on the aldehyde mutase which will be shortly described in detail, it was found that ammonium sulphate inhibits and also partly destroys irreversibly the aldehyde mutase but leaves the alcohol dehydrogenase unaffected.

Methylene blue in concentrations suitable as a carrier for the alcohol dehydrogenase slows down considerably the mutase activity.

Animal tissues other than liver

When tested by the methods described in this paper, positive results were obtained with alcohol dehydrogenase preparations from intestine and kidney, but brain and muscle gave negative results.

Discussion

The mechanism of the oxidation of alcohol in yeast has been much studied and recently Negelein & Wulff [1937] in Warburg's laboratory have isolated in crystalline form the alcohol dehydrogenase from yeast. However, the alcohol dehydrogenase of animal tissues has received much less attention in spite of its occurrence in various animal tissues. From the experimental evidence given in this paper several conclusions can be drawn regarding the properties and the behaviour of the animal alcohol dehydrogenase under various conditions.

It has been shown that the enzyme is able to oxidize alcohol both anaerobically and aerobically but it requires for its activity the presence of some additional substances. The coenzyme necessary for this reaction is cozymase, and this property seems to be general for all enzyme systems concerned with alcohol oxidation (yeast, animal tissues, bacteria and also probably plants).

Acetone-treated enzyme preparations require for their completion under anaerobic conditions the addition of the coenzyme factor, so that this factor must be regarded as an essential part of the enzyme system. The omission of the coenzyme factor led in older observations to the unjustified view that the enzyme is particularly unstable.

In tissue slices [Leloir & Munoz, 1938] or in liver extracts alcohol undergoes rapid oxidation, but the acetone- or ammonium sulphate-treated enzyme preparations react with O_2 slowly and only if a carrier such as methylene blue is provided. The fact that an acceleration of O_2 uptake can be achieved through heart muscle cytochrome oxidase suggests the probability of a similar process actually taking place when alcohol is oxidized in the intact liver tissue. It also explains why it has been frequently assumed that the alcohol oxidase system from animal tissues is a CN-sensitive system. We found that under anaerobic conditions cyanide either has no effect upon the enzyme or slightly accelerates the reaction, probably by fixing the aldehyde. Thus the cyanide inhibition observed is due solely to the poisoning of the cyanide-sensitive cytochrome oxidase system.

It is difficult to regard flavoprotein as a part of the animal alcohol dehydrogenase system. It has only a very small effect on the course of the reaction, when added to preparations repeatedly treated with acetone.

When alcohol is oxidized anaerobically by the enzyme preparation either alone or with the addition of a washed muscle suspension as coenzyme factor, acetaldehyde is found as the reaction product. It is difficult to account for the fact that no acetaldehyde is found when washed liver suspensions are used as the source of the coenzyme factor. Such washed liver suspensions alone show no aldehyde dehydrogenase or mutase activity, and when acetaldehyde is added and incubated with the experimental mixture composed of the liver enzyme preparation, liver suspension (coenzyme factor), cozymase and methylene blue, it is recovered quantitatively. The possibility remains of the alcohol dehydrogenation being directed into a hitherto unknown route in presence of the washed liver suspension, or of its being linked with some secondary reaction. But to prove this assumption purification and closer analysis of the liver suspension is necessary.

In experiments in which the course of the reaction is very slow, e.g. in aerobic conditions or anaerobically without additional coenzyme factor, the yield of acetaldehyde is smaller than would be expected from the amount of alcohol added and some acetic acid is produced. This can be accounted for by the presence in the preparations of aldehyde mutase and in some of the more crude preparations also of aldehyde oxidase. It explains why in the intact liver tissue acetic acid is found almost exclusively as the product of alcohol oxidation.

In contrast to the yeast alcohol dehydrogenase, the animal (and also the bacterial) alcohol dehydrogenase seems to be only little affected by iodoacetate, even if large concentrations of the iodoacetate are allowed to act upon the enzyme for some time. This finding, together with the fact that flavoprotein does not seem to be a constituent of the animal enzyme, suggests the possibility of some essential difference between the structures of the yeast and the animal alcohol dehydrogenases.

Dixon & Lutwak-Mann [1937] have shown that by suitable methods of preparation and purification it is possible to obtain the liver aldehyde mutase free from alcohol dehydrogenase. The present results, where the actions of various inhibitors were tested both on the alcohol dehydrogenase and on the mutase, furnish further evidence that these two enzymes are distinct systems.

Summary

Various methods of preparation of the liver alcohol dehydrogenase and its properties are described.

By using suitable preparations it can be shown that cozymase and cozymase factor are essential components of the system.

According to the treatment, preparations can be obtained which contain no cozymase but have enough coenzyme factor (precipitation with ammonium sulphate) and vice versa (precipitation with acetone). The alcohol dehydrogenase in the preparations remains unaltered over a period of months.

Under certain experimental conditions acetaldehyde is obtained quantitatively as the reaction product of alcohol oxidation. The probability of other courses of alcohol oxidation is discussed.

The alcohol dehydrogenase in the preparations cannot react directly with O_2. The presence of carriers (methylene blue, pyocyanine) is necessary. The reaction with O_2 is markedly accelerated by addition of cytochrome oxidase preparations.

Ethyl, propyl, methyl and amyl alcohols are oxidized by the liver alcohol dehydrogenase preparations. With glycerol, α-glycerophosphate and saligenin negative results are obtained.

Iodoacetate has little effect upon the animal alcohol dehydrogenase. The enzyme is not sensitive to cyanide. Evidence is provided for the non-identity of the liver alcohol dehydrogenase and aldehyde mutase.

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CLXXXII. THE MOLECULAR WEIGHT OF CROTOXIN

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(*Received 20 June 1938*)

Slotta & Fraenkel-Conrat [1938, 1] have succeeded in purifying and crystallizing the active substance in the venom from the rattlesnake (*Crotalus terrificus*). They showed that this snake poison is a protein, with a large sulphur content (4%), and gave it the name crotoxin. As far as we know, this is the first snake poison ever isolated in a pure crystalline state. It was therefore of great interest to determine its molecular weight.

From analytical determinations of the methionine content of the protein molecule, Slotta & Forster [1938] conclude that the minimum molecular weight is 11,000. They state that it is very probable that the real size is three or six times this value.

We have determined the molecular weight of crotoxin by the ultracentrifugal methods developed in this laboratory as described by Svedberg [1937].

The partial specific volume of the crotoxin was found to be 0·704, which is a comparatively low value for a protein. Due to lack of material we were not able to make more than one determination (1% protein in 4% salt solution). However, the error should not exceed 2%.

The sedimentation constant was determined in the ultracentrifuge at a speed of 70,000 r.p.m., corresponding to a centrifugal force of 350,000 *g*. The observations were made by the scale method of Lamm [1937]. All the solutions were prepared immediately before the experiments from a stock solution, containing 1% crotoxin in 0·67 *M* NaCl. Table I gives the results.

Table I. *Sedimentation velocity determinations*

Crotoxin concentration %	Salts in solvent	Total salt molarity	*p*H	s_{20}*
1·0	NaCl	0·67	Not buffered	3·10
0·5	,,	0·67	,,	3·18
0·3	,,	0·2	,,	3·09
0·2	,,	0·2	,,	3·14
0·3	NaCl, HOAc	0·25	3·8	2·92
0·3	,,	0·25	3·8	2·95
0·3	NaCl, HOAc, NaOAc	0·25	4·4	2·92
0·3	,,	0·26	5·5	3·06
0·3	NaCl, KH_2PO_4, $Na_2B_4O_7$	0·23	8·7	3·21
0·3	NaCl, $Na_2B_4O_7$, Na_2CO_3	0·25	10·0	3·20

* Sedimentation constants are given in units of 10^{-13}.

The sedimentation constant is, within experimental error, independent of concentration. The average of the first four determinations is $s_{20}=3{\cdot}13$. In acid solutions the sedimentation constant is a little lower, but in neutral and alkaline solutions it has about the same value. The sedimentation curves obtained are symmetrical and suggest a homogeneous substance.

The diffusion of crotoxin was measured according to Lamm's method [1937; see also Lamm & Polson, 1936]. The following results were obtained:

Table II. *Diffusion determinations*

Crotoxin concentration %	Salts in solvent	Total salt molarity	D_{20}*
1·0	NaCl	0·67	8·70
0·6	NaCl, Na_2HPO_4, KH_2PO_4	0·72	8·51
0·5	NaCl	0·67	8·55
0·25	NaCl	0·67	7·55

* Diffusion constants are given in units of 10^{-7}.

The average of the first three values is $D_{20}=8{\cdot}59$. The last value is probably not so accurate, owing to the low concentration. The diffusion curves are in all experiments very nearly normal distribution curves, indicating a homogeneous substance. The diffusion constant was calculated also from the spreading of the boundary in the first of the sedimentation experiments, which gave $D_{20}=9{\cdot}0$. Such a calculation cannot give the same accuracy as a static diffusion experiment, but its good agreement with the figures in Table II shows that crotoxin is a monodisperse protein.

Using the formula

$$M=\frac{RT\,s_{20}}{(1-\bar{V}\rho)D_{20}}$$

a value of 30,000 is obtained for the molecular weight of crotoxin. The ratio of the molecular frictional constant to that of a spherical molecule of the same weight, f/f_0, is 1·2. Slotta's value of 11,000 for the minimum molecular weight is, within experimental errors, one-third of our value.

To check the value we have also made a sedimentation equilibrium determination. The formula used for calculation is

$$M=\frac{2RT\ln(c_2/c_1)}{(1-\bar{V}\rho)\,\omega^2\,(x_2^2-x_1^2)}.$$

The speed of the centrifuge was 10,000 r.p.m. Two cells were used, placed at 5·0 and 5·7 cm. distance from the centre of rotation. They both gave the same average value of 30,500 for the molecular weight. There was no drift in the calculated values along the radius, showing that the substance is homogeneous. This is in agreement with the results of the diffusion measurements given above.

Slotta & Fraenkel-Conrat [1938, 2] have found that cysteine reduces the poison value (for definition see Slotta & Szyszka [1938]) of the crotoxin, probably due to its specific reduction power for protein-S—S-bonds. It was of interest to see whether it was possible to determine the particle size of the decomposition products. We therefore made an experiment according to Slotta's & Fraenkel-Conrat's experimental data. The only difference was that for ultracentrifugal determinations we needed a higher concentration of the protein than they used. We started with a 0·25 % solution of crotoxin; on standing with cysteine a precipitate was formed, which was probably cystine. The solution remaining was ultracentrifuged, and was found to contain very little high-molecular material, but consisted mainly of low-molecular products of variable, undeterminable size.

Crotoxin, which had been standing 3 days at 37° and *p*H 8·7, contained the usual high-molecular component (s_{20} was measured in two experiments, 3·39 and 3·08), but in addition decomposition products of low molecular weight were

present. It is of interest to note that Slotta has found a decrease in the poison value by this treatment.

It is therefore obvious that the poison activity is associated with the unchanged protein, as already pointed out by Slotta, and the decomposition products have no, or only a very small, activity, as is observed also with the decomposition products of enzymes and other active proteins.

Summary

1. The partial specific volume of crotoxin was determined to be $V=0{\cdot}704$.
2. The sedimentation constant of crotoxin was found to be independent of concentration and of *p*H within the range 5·5–10·0. Its value was 3·13.
3. The diffusion constant was found to be 8·59.
4. Crotoxin behaved in sedimentation and diffusion as a homogeneous substance, and the molecular weight was calculated to be 30,000. The frictional ratio is 1·2.
5. Sedimentation equilibrium measurements gave the value 30,500 for the molecular weight, in good agreement with the preceding value.
6. The molecule is split into smaller, inactive substances by treatment with cysteine or by warming in alkaline solution.

The authors' thanks are due to Prof. K. H. Slotta for suggesting the problem and for his kindness in supplying 120 mg. crystalline crotoxin for the investigation. The expenses connected with the work were defrayed by grants from the Andersson Foundation.

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CLXXXIII. THE *l*(+)GLUTAMIC DEHYDROGENASE OF ANIMAL TISSUES

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THUNBERG [1920] observed that natural glutamic acid was oxidized in the presence of frog muscle. Weil-Malherbe [1936] was the first to prepare an enzyme extract which catalysed this oxidation but he did not resolve the system into its catalytic components.

While this present analysis of the *l*(+)glutamic dehydrogenase system was in progress Adler *et al.* [1937] described the properties of the same enzyme in liver. They found that coenzyme I was essential to the system and that the products of the reaction were α-ketoglutaric acid and ammonia. They also demonstrated the reversibility of the reaction.

I. *Preparation of the components*

The *l*(+)glutamic dehydrogenase was prepared from liver or kidney of pig as follows. About 300 g. liver or kidney are minced in a Latapie mincer. To the mince are added 4 vol. cold acetone and the mixture is stirred for about 3 min., filtered on a Büchner funnel and washed in turn with acetone and ether. The material on the filter paper is again mixed with 4 vol. acetone, filtered, washed with acetone and ether and spread out on paper to dry in a current of air. 10 g. of the dry powder are rubbed up with 100 ml. distilled water and the mixture centrifuged. The supernatant fluid after filtration is acidified with 10% acetic acid to *p*H 4·6, the centrifuged precipitate washed with distilled water and finally suspended in 25 ml. *M*/10 phosphate buffer, *p*H 7·3. The enzyme keeps its activity in solution at 0° for 4–5 days. The acetone powder can be kept *in vacuo* for several weeks without loss of activity.

Coenzyme I was prepared by the method outlined by Green *et al.* [1937].

II. *The reaction with molecular oxygen*

A mixture of the dehydrogenase, coenzyme I and *l*(+)glutamic acid in the presence of a carrier such as pyocyanine takes up O_2 readily. The rate of O_2 uptake is dependent on the concentrations of the various reactants, i.e. dehydrogenase, coenzyme, substrate and carrier (cf. Figs. 1–4). As in other coenzyme systems the proportionality between the rate of oxidation and the concentrations of the different components holds only at low concentrations. Above some limiting concentration the system becomes saturated with the component in question.

The Michaelis constant, i.e. the substrate concentration at which half the maximum velocity is reached is approximately *M*/133 (Fig. 3).

Carriers which have been found to be active in the *l*(+)glutamic acid system include pyocyanine, methylene blue, cytochromes *a* and *b*, flavinphosphate and flavoprotein from yeast (Table I). Cytochrome *c* in large concentrations (1·2 mg./ml.) has a small effect on the O_2 uptake. The most efficient carrier

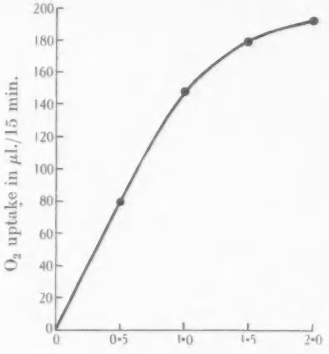

Fig. 1.

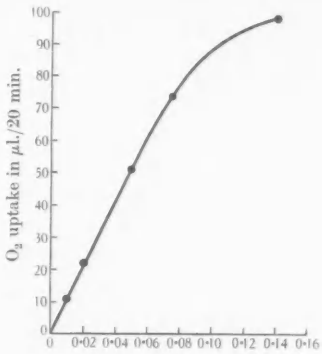

Fig. 2.

Fig. 1. The effect of the concentration of dehydrogenase. Each manometer contained 1·0 ml. 0·3% coenzyme I, 0·2 ml. 0·1% pyocyanine and 0·5 ml. *M*/3 *l*(+)glutamic acid.

Fig. 2. The effect of the concentration of coenzyme I. Each manometer contained 1·5 ml. enzyme, 0·2 ml. 0·1% pyocyanine and 0·5 ml. *M*/3 *l*(+)glutamic acid.

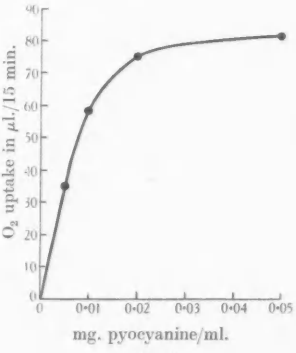

Fig. 3.

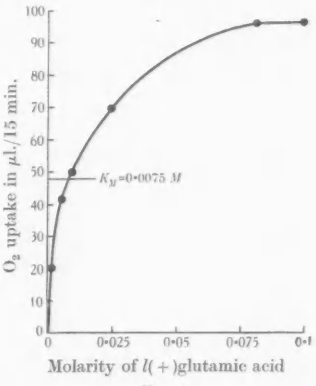

Fig. 4.

Fig. 3. The effect of the concentration of pyocyanine. Each manometer contained 1·5 ml. enzyme, 1·0 ml. coenzyme I and 0·5 ml. *M*/3 *l*(+)glutamic acid.

Fig. 4. The effect of the concentration of substrate. Each manometer contained 1·0 ml. enzyme, 1·0 ml. 0·3% coenzyme I and 0·2 ml. 0·1% pyocyanine. The total volume was 3·3 ml.

Table I. *The effect of carriers on the reaction with O_2*

The system contained 1·5 ml. enzyme; 1·0 ml. 0·3% coenzyme and 0·5 ml. *M*/3 *l*(+)glutamic acid. The total volume was 3·3 ml.

	μl. O_2 in 15 min.
l(+)glutamic system without carrier	0
System + 1·0 mg. methylene blue	56
System + 0·2 mg. pyocyanine	84
System + 0·5 mg. flavinphosphate	24
System + 2·5 mg. pure flavoprotein (yeast)	56
System + 0·3 ml. cytochromes *a* and *b* preparation	125

system was the cytochrome *a* and *b* system of Keilin and Hartree (cf. Dewan & Green [1938] for method of preparation). It has already been shown in other coenzyme systems that the coenzyme factor rapidly catalyses the oxidation of reduced coenzyme by cytochromes *a* and *b* [Dewan & Green, 1938].

M/100 cyanide inhibited the *l*(+)glutamic system when the cytochromes were used as carriers (Table II). The inhibition was practically complete during the first 5 min. but became progressively less as the reaction proceeded owing to the accumulation of α-ketoglutaric acid and subsequent cyanohydrin formation. *M*/500 cyanide reduced the rate of O_2 uptake by about 66% in the first 5 min. Here again the extent of inhibition decreased as the reaction progressed. Cyanide even in *M*/10 concentration had no inhibitory effect when pyocyanine was used as carrier.

Table II. *The effect of cyanide on the* l(+)*glutamic system with cytochromes* a *and* b *as carriers*

The complete system contained 1·5 ml. enzyme, 1·0 ml. 0·3% coenzyme I, 0·5 ml. preparation of cytochromes *a* and *b* and 0·3 ml. *M*/3 *l*(+)glutamic acid.

	μl. O_2 in 5 min.	μl. O_2 in 10 min.
Complete *l*(+)glutamic system	30	53
With *M*/500 HCN	11	25
With *M*/100 HCN	0	5

Ketone fixatives such as hydrazine, semicarbazide and cyanide were tested for their effect on the velocity of oxidation of *l*(+)glutamic acid. Hydrazine had no appreciable effect. Semicarbazide and cyanide did not influence the

Table III. *The effect of fixatives*

The system contained 1·5 ml. enzyme, 1·0 ml. coenzyme, 0·2 ml. 0·1% pyocyanine, 0·4 ml. *M*/3 *l*(+)glutamic acid. The fixative solutions were neutralized.

	μl. O_2		
	10 min.	20 min.	30 min.
l(+)Glutamic system	142	200	226
+0·2 ml. *M*/2 semicarbazide	157	222	260
+0·2 ml. 2 *M* HCN	162	246	300

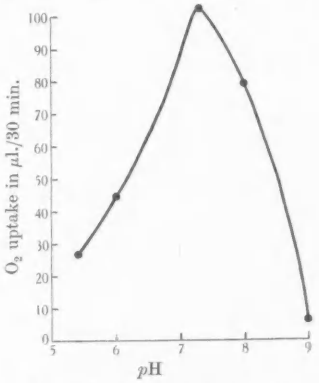

Fig. 5. The effect of *p*H. Each manometer contained 1·0 ml. enzyme, 0·8 ml. coenzyme, 0·2 ml. 0·1% pyocyanine and 0·3 ml. *M*/3 *l*(+)glutamic acid.

initial velocity but had a slight effect in preventing falling off in velocity as the reaction proceeded (cf. Table III). α-Ketoglutaric acid inhibits the oxidation of *l*(+)glutamic only when present in high concentration. This indicates that the glutamic system is much more negative in potential than the coenzyme system.

Fig. 5 shows the dependence of the rate of oxidation of *l*(+)glutamic acid on the *p*H of the solution. The maximum velocity is observed at about *p*H 7·3, the rate falling off rapidly above and below this value.

Effect of coenzyme factor

The presence of an enzyme in animal tissues which catalyses the oxidation of reduced coenzyme by carriers was demonstrated by Dewan & Green [1938] and Adler *et al.* [1937]. Coenzyme factor is also necessary in the *l*(+)glutamic system. To obtain a dehydrogenase preparation free from the factor, pig kidney, after mincing in a Latapie mincer, was allowed to stand 30 min. in 2 vol. iced water and pressed through muslin. The filtrate was mixed with 3 vol. cold acetone, the precipitate washed with acetone and ether and rubbed up in a mortar until dry. The acetone powder was then treated in the usual manner of preparing the *l*(+)glutamic dehydrogenase. The enzyme solution before using was filtered through kieselguhr and charcoal to remove last traces of coenzyme factor. The factor was prepared from rabbit skeletal muscle [cf. Dewan & Green, 1938].

Table IV shows that coenzyme factor is necessary for the aerobic oxidation of *l*(+)glutamic acid in presence of dehydrogenase, coenzyme I and pyocyanine.

Table IV. *Effect of coenzyme factor*

The complete system contained 0·5 ml. enzyme, 1·0 ml. coenzyme, 0·05 ml. 0·1% pyocyanine, 1·0 ml. factor and 0·5 ml. *M*/3 *l*(+)glutamic acid.

	μl. O_2 in 30 min.
System	31
Without factor	3
Without enzyme	0
Without glutamic acid	0

III. *The products of oxidation*

l(+)Glutamic acid on oxidation yields α-ketoglutaric acid and NH_3. The ketonic acid was isolated and identified as follows. A mixture containing 100 ml. enzyme, 50 ml. 0·3% coenzyme I, 10 ml. 0·1% pyocyanine and 25 ml. *M*/3 *l*(+)glutamic acid was aerated vigorously at 37° for 90 min., deproteinized with 50% trichloroacetic acid and filtered. The filtrate was concentrated *in vacuo* to 25 ml. 1 g. 2:4-dinitrophenylhydrazine in 100 ml. 2*N* HCl was added to the filtrate when crystals of the 2:4-dinitrophenylhydrazone began forming at once. The mixture was left at 0° for 10 hr. The precipitate was washed with 2*N* HCl and dried. Just sufficient ethyl acetate was added to dissolve the crystals and a small brown residue was filtered off. To the filtrate were added 4 vol. ligroin. The precipitate was centrifuged, washed with ligroin and finally dried *in vacuo*. M.P. 218°; M.P. of synthetic α-ketoglutaric acid 2:4-dinitrophenylhydrazone 218°; mixed M.P. 218°. Found (Weiler): C, 40·81%; H, 3·31%; N, 17·1%. $C_{11}H_{10}O_8N_4$ requires C, 40·44%; H, 3·09%; N, 17·18%.

The ratio of α-ketoglutaric acid formed to O_2 absorbed was determined by estimation of the $NaHSO_3$-binding of the deproteinized system after recording the O_2 uptake. Table V shows that for every atom of oxygen absorbed, approximately 1 mol. α-ketoglutaric acid was formed.

Table V. *Estimation of α-ketoglutaric acid formed by oxidation of l(+)glutamic acid*

The complete system contained 1·5 ml. enzyme, 1·0 ml. coenzyme, 0·2 ml. 0·1% pyocyanine and 0·5 ml. *M*/3 *l*(+)glutamic acid. Control had no substrate.

mg. α-ketoglutaric acid found by measuring $NaHSO_3$-binding power	2·2
μl. O_2 absorbed during the oxidation of the *l*(+)glutamic acid	172
Theoretical amount (in mg.) of α-ketoglutaric acid, assuming 1 mol. α-ketoglutaric acid is produced for each atom O absorbed	2·25

Further confirmation that 1 atom O is absorbed for each mol. *l*(+)glutamic acid oxidized is obtained from the O_2 equivalence of small amounts of *l*(+)-glutamic acid. Table VI shows the close agreement between the O_2 uptake for known amounts of *l*(+)glutamic acid and the theoretical uptake, calculated on this basis. With larger amounts of substrate the O_2 uptake was somewhat lower than the theoretical value, due to the fact that enzymic activity ceases before the whole of the glutamic acid is oxidized.

Table VI. *The O_2 equivalence of* l(+)*glutamic acid*

Each manometer contained 2·0 ml. enzyme, 1·0 ml. coenzyme I and 0·2 ml. 0·1% pyocyanine. The substrate was placed in Keilin cups which were introduced into the main body of the fluid after equilibration. The control O_2 uptake without substrate has been subtracted from the experimental values.

Millimol. *l*(+)glutamic acid	0·25	0·5	0·75	1·0
μl. O_2 absorbed	26	58	81	94
Theoretical uptake assuming that 1 atom O reacts with 1 mol. glutamic acid	28	56	84	112

NH_3 estimations. The O_2 uptake of the complete glutamic system was measured manometrically. A control experiment containing the whole system with the exception of substrate was carried out simultaneously. After a run of 90 min. NH_3 was estimated by the Parnas method. Table VII shows that the amount of NH_3 found correlates well with the theoretical value calculated from the O_2 uptake.

Table VII. *NH_3 estimations*

Complete system contained 1·0 ml. enzyme, 1·0 ml. coenzyme, 0·2 ml. 0·1% pyocyanine and 0·5 ml. *M*/3 *l*(+)glutamic acid.

mg. NH_3 found (corrected for control)	Theoretical NH_3 assuming that for 1 atom O absorbed 1 mol. NH_3 is produced	% theory
(1) 0·17	0·18	94
(2) 0·26	0·28	92

IV. *Specificity of substrate*

The glutamic dehydrogenase specifically catalyses the oxidation of *l*(+)-glutamic acid to α-ketoglutaric acid and NH_3. The unnatural isomeride is not oxidized.

dl-β-Hydroxyglutamic acid was attacked at one-tenth the rate of *l*(+)glutamic acid. Alanine, phenylalanine, valine, histidine and leucine of the *l*-series are not oxidized in the presence of the dehydrogenase preparation. Some preparations catalysed the oxidation of *l*-aspartic acid and *l*-cystine but these oxidations were found to be unconnected with the glutamic dehydrogenase. A sample of glutamine showed slight activity, but as it contained small amounts of glutamic acid it was difficult to assess the results.

V. *Specificity of coenzyme*

Coenzyme I cannot be replaced by coenzyme II in the *l*(+)glutamic system (cf. Table VIII). Coenzyme II was prepared from horse red blood cells and its activity checked with the hexosemonophosphate system.

Table VIII. *Specificity of coenzyme*

Experiments were carried out under anaerobic conditions in Thunberg tubes. The *l*(+)glutamic system contained 2 ml. enzyme, 0·2 ml. 0·5% methylene blue and 0·5 ml. of *M*/3 *l*(+)glutamic acid. The Thunberg tubes after evacuation were immersed in a water bath at 38°.

System	Reduction time of methylene blue min.
	∞
+0·15 mg. coenzyme I	5
+0·15 mg. coenzyme II	∞

VI. *Reduction of coenzyme I*

Coenzyme I was reduced by *l*(+)glutamic acid in the presence of the dehydrogenase (cf. Table IX). The reduction was demonstrated spectrophotometrically by the appearance of the characteristic band of reduced coenzyme with a peak at 340 mμ.

Table IX. *Reduction of coenzyme I by* l(+)*glutamic acid in presence of the dehydrogenase*

The system contained 1·0 ml. 0·3% oxidized coenzyme I, 0·3 ml. *l*(+)glutamic acid and 3·1 ml. 0·25% $NaHCO_3$. Total vol. 8 ml. Incubated in Thunberg tubes at 38° for 5 min. Contents then boiled and filtered through kieselguhr. The clear filtrate was used directly for analysis in the Hilger Spekker spectrophotometer [cf. Green & Dewan, 1937]. Two controls were done, one containing the system without *l*(+)glutamic acid, the other without the dehydrogenase.

System	log I_0/I at 340 mμ
System	0·7
System without *l*(+)glutamic acid	0·1
System without dehydrogenase	0·1
Oxidized coenzyme	0·1

VII. *Distribution*

Various tissues of pig were investigated for the presence of *l*(+)glutamic dehydrogenase. Acetone powders of the tissues were made in the usual way. 4 g. of the powder in each case were ground up in a mortar with 40 ml. distilled water, centrifuged, the supernatant liquid filtered from fat and precipitated with 10% acetic acid at *p*H 4·6. The sediment was washed with distilled water and resuspended in 10 ml. *M*/10 phosphate buffer, *p*H 7·3. The system contained 2 ml. enzyme, 1·0 ml. 0·3% coenzyme I, 0·2 ml. 0·1% pyocyanine and 0·5 ml. *M*/3 *l*(+)glutamic acid. A control containing the system without *l*(+)glutamic acid was carried out in each case and the O_2 uptake subtracted from the value obtained with the complete system.

	μl. O_2 in 30 min.
System using liver enzyme	274
System using kidney enzyme	270
System using heart enzyme	25

The enzyme was detected in muscle and brain but in concentrations too small for accurate measurements.

VIII. *Reversibility*

The reaction between glutamic acid and coenzyme I may be formulated as follows:

$$l(+)\text{Glutamic acid} + \text{coenzyme I} \rightarrow \alpha\text{-ketoglutaric acid} + NH_3 + \text{reduced coenzyme I.}$$

If this reaction is reversible it should be possible to obtain glutamic acid from a mixture of the reduced coenzyme I, NH_3 and α-ketoglutaric acid. Reduced coenzyme I can be obtained either enzymically or by reduction with hyposulphite. In the following procedure the β-hydroxybutyric system was used to reduce the coenzyme, and the production of acetoacetic acid under anaerobic conditions was used as a measure of the reaction between reduced coenzyme on the one hand, and NH_3 and α-ketoglutaric acid on the other. For the theory of this method, cf. Dewan & Green [1937] on coenzyme-linked reactions. Acetoacetic acid was measured by the aniline citrate method of Ostern [1933]. The enzyme was prepared from pig's heart [cf. Green *et al.* 1937]. This preparation contains dehydrogenases for both *l*-β-hydroxybutyric acid and *l*(+)glutamic acid. Table X shows that the reversed reaction takes place when all components of both systems are present. The small blank in the absence of NH_4Cl is due to the reversibility of the α-hydroxyglutaric dehydrogenase system which has been shown by Weil-Malherbe [1937] to be present in heart.

Table X. *Production of acetoacetic acid in the reaction between β-hydroxybutyric acid, α-ketoglutaric acid and NH_3*

The complete system contained 0·2 ml. *M dl*-β-hydroxybutyric acid, 0·8 ml. *M*/3 NH_4Cl, 0·2 ml. *M*/6 α-ketoglutaric acid, 1·5 ml. heart enzyme preparation and 1 ml. coenzyme I. The substrates were neutralized before using.

	CO_2 production in 60 min.
Complete system	190
Without *dl*-β-hydroxybutyric acid	0
Without NH_4Cl	27
Without α-ketoglutaric acid	0
Without heart enzyme preparation	0
Without coenzyme I	0

Further proof of the reversibility of the *l*(+)glutamic system is derived from the demonstration of the formation of amino-N when all the components of the coenzyme-linked reaction were present. Three mixtures were set up in Thunberg tubes under anaerobic conditions; one contained the complete system, a second was without NH_4Cl and a third without α-ketoglutaric acid. After 1–2 hr. incubation at 38° the contents of each tube were deproteinized and the filtrates made up to the same final volumes. Acetoacetic acid estimations were made on one half of the filtrates and amino-N (Van Slyke method) on the other half.

Table XI. *Production of amino-N in the reaction between β-hydroxybutyric acid, α-ketoglutaric acid and NH_3*

Complete system contained 3·0 ml. heart enzyme preparation, 1·5 ml. 0·3% coenzyme I, 0·2 ml. *dl*-hydroxybutyric acid, 1·0 ml. *M*/3 NH_4Cl and 0·2 ml. *M*/6 α-ketoglutaric acid. Two controls were done, one without NH_4Cl, the other without α-ketoglutaric acid.

	(1)	(2)
Amino-N found	0·04 mg.	0·088 mg.
Theoretical amount of amino-N expected from CO_2 production assuming that 1 atom amino-N is formed for each mol. CO_2 produced.	0·05 mg.	0·081 mg.

The small amino-N values in the controls were averaged and this value was subtracted from the amount obtained in the complete system. Table XI shows that the amino-N found agrees well with the theoretical amount expected from the acetoacetic acid production on the assumption that 1 atom amino-N is formed for each mol. acetoacetic acid produced.

Summary

1. The preparation and properties of *l*(+)glutamic dehydrogenase of animal tissues are described. Coenzyme I is a necessary component of the system.

2. The dehydrogenase catalyses the oxidation of *l*(+)glutamic acid to α-ketoglutaric acid and NH_3. The reversibility of this reaction has been demonstrated.

I should like to express my thanks to Prof. Sir F. G. Hopkins for the interest he has shown in the progress of this work and to Dr D. E. Green for his constant advice, to Prof. C. R. Harington for the gift of a sample of *dl*-β-hydroxyglutamic acid and to Mr S. Williamson for some of the chemical preparations and the amino-N estimations.

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CLXXXIV. THE PURIFICATION OF URICASE

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In the course of an attempt to isolate the enzyme uricase, which oxidizes uric acid to allantoin, it has been found possible to obtain a preparation 550 times purer than the liver powder which is used as starting material [Davidson, 1938]. The purest preparation contains 0·15–0·20 % Fe, a trace of Cu, no Co or Mn and 14·4 % N. Small quantities are colourless, and even very large quantities have only a very pale brown colour. It is therefore not a haemin derivative. It is quite insoluble in water, almost insoluble in phosphate buffer *p*H 7·4 but soluble in alkaline solution, e.g. borate buffer *p*H 10. Truszkowski [1934] and Ro [1931] have already shown that uricase is soluble in alkaline solution.

In order to determine the enzymic activity a manometric test has been developed. The main chamber of the manometer vessel contains 1·0 ml. *M*/5 borate buffer *p*H 9 and 1·5 ml. enzyme preparation plus water. The potash tube contains 0·2 ml. 10 % KOH, and the side bulb 2·24 mg. uric acid as *M*/30 lithium urate. (0·56 g. uric acid is dissolved in 35 ml. boiling *N*/10 lithium hydroxide and the solution is made up to 100 ml. with water. This solution must be freshly prepared each day.) The gas space contains pure oxygen and the oxygen uptake is measured at 38° over a period of 30 min.

By the specific activity of the enzyme is meant the number of μl. O_2 which are taken up per min. in the first 30 min. by 1 mg. of dry enzyme preparation. The specific activity of the best preparation is 85–90 μl. per mg. per min. as compared with a value of 0·12–0·15 μl. per mg. per min. for the dry liver powder from which it is obtained.

The most convenient starting material has been found to be pig's liver, which has already been used by Kleinmann & Bork [1933] and by Keilin & Hartree [1936] for uricase studies. The pig's liver is finely minced and ground in a mortar with five parts of acetone. The solid material is centrifuged off and shaken with three parts of acetone. After centrifuging, the liver powder is dried at first rapidly in a current of air and then overnight in vacuum desiccators, and is then finely powdered and passed through a sieve. The resulting light brown powder has a specific activity of 0·12–0·15 μl. per mg. per min. and retains its activity for several months when stored in desiccators at room temperature. Samples showing a specific activity of less than 0·10 μl. per mg. per min. are discarded.

250 g. liver powder are stirred with 2·5 l. ice-cold *M*/10 phosphate buffer *p*H 7·4. After standing for 20 min. at 0° the mixture is centrifuged, and the extract, which contains much protein but only little uricase is discarded. The solid residue is then stirred with 5 l. *M*/10 borate buffer *p*H 10 at 38°. After 20 min. the mixture is rapidly cooled to 0° and centrifuged. The clear extract contains the bulk of the enzyme and has a specific activity of 0·5 μl. per mg. per min.

An equal volume of saturated ice-cold ammonium sulphate solution is added to this solution, and the resulting precipitate, which contains the enzyme, is centrifuged off at 0° and dissolved with shaking in 5 l. ice-cold distilled water.

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1/10 vol. saturated ice-cold ammonium sulphate is then added (no precipitate being formed), and the solution, which has a *p*H of 7·2–7·4, is heated to 55° for 5 min. with vigorous stirring, whereby a heavy flocculent precipitate of denatured protein is formed. The solution is rapidly cooled to 0° and the precipitate is centrifuged off and discarded. The enzyme solution, which now has a specific activity of 1·0–1·5 μl. per mg. per min., is treated with enough saturated ammonium sulphate solution at 0° to bring the degree of saturation to 0·5. The resulting precipitate, which contains the enzyme, is centrifuged off at 0° and dissolved in 1 l. of ice-cold distilled water. This solution is dialysed for 15 hr. in cellophane sacs against running distilled water at 0°. A brown precipitate which appears during the dialysis and which contains the enzyme is centrifuged off and washed with ice-cold water on the centrifuge. It is then rubbed in a cooled mortar with 400 ml. *M*/10 phosphate buffer *p*H 7·4 at 0° and centrifuged on the high speed centrifuge (15,000 r.p.m.). The extract, which contains much protein but little enzyme, is discarded. The residue is rubbed in a mortar with 250 ml. *M*/10 borate buffer *p*H 10 at room temperature and is centrifuged on the high speed centrifuge. The extraction with 250 ml. borate buffer is repeated, the extracts, which contain the bulk of the enzyme, are combined, and the residue, which consists of brown insoluble protein, is discarded.

The clear, pale yellow uricase solution which now has a specific activity of 10–25 μl. per mg. per min. is cooled to 0°, treated with an equal volume of saturated ice-cold ammonium sulphate solution, and the resulting precipitate, which contains the enzyme, is centrifuged off at 0° and dissolved in 60 ml. ice-cold water.

To the solution just enough (0·5–1·0 vol.) ice-cold 80% acetone is added so that a flocculent precipitate appears after 20 min. The precipitate is centrifuged off and discarded. Acetone is removed from the supernatant fluid by vacuum distillation at 20° with two drops octyl alcohol and the resulting slightly turbid solution, after standing overnight at 0°, is centrifuged on the high speed centrifuge. A small precipitate of very high activity is thus obtained, and is washed three times on the centrifuge with ice-cold distilled water. It is then rubbed up with 5 ml. *M*/10 borate buffer *p*H 10 at room temperature, in which the enzyme dissolves, leaving a small inactive brownish residue which is centrifuged off at high speed and discarded.

The uricase solution is almost colourless. When it is dialysed against running distilled water at 0° the free enzyme is obtained. Specific activity 85–90 μl. per mg. per min. The yield of the purest preparation is about 5 mg. per 100 g. liver powder.

On standing the activity of the free enzyme gradually diminishes but in solution at *p*H 10 it is stable for several weeks. Some of the enzyme frequently precipitates out in an insoluble form. The enzyme cannot be dried without great loss of activity.

The velocity of the test reaction is dependent on the oxygen pressure and in a mixture of 2% oxygen and 98% argon it is only 7% as great as 100% oxygen. If the argon is replaced by carbon monoxide no inhibition by the carbon monoxide is found.

Very small quantities of cyanide, however, inhibit the reaction reversibly, as has been already pointed out by Keilin & Hartree [1936] and by Truszkowski [1930]. The enzyme therefore would appear to be a heavy metal compound, but it has nevertheless not yet been proved that the catalytic activity of the enzyme is due to the iron. Attempts to remove iron by dialysis with cyanide after the manner in which Kubowitz [1938] removed copper from phenoloxidase, and by

other methods, have not so far been successful. The colourless nature of the enzyme suggests that the iron is not present in the form of a haemin derivative. On the other hand the comparatively large amount of iron (of the same order as that found by Sumner & Dounce [1937] in crystalline catalase) and the consistency with which the value 0·15–0·20 % is found in repeated preparations, suggests that the iron is more than a mere impurity.

If it is assumed that the catalytic activity of the enzyme is due to the iron, then 1 mg. iron brings about the reaction of 57,000 μl. oxygen per min.

Summary

A method for the purification of the enzyme uricase is described.

The purest preparation has a specific activity, under stated conditions, of 85–90 μl. per mg. per min. and contains 0·15–0·20 % Fe.

Although uricase seems to be a heavy metal compound it has not yet been proved that the iron acts as the active group of the enzyme.

I should like to express my thanks to Prof. Otto Warburg for his generous provision of laboratory facilities and for his continued interest and encouragement during the course of this work.

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CLXXXV. GLYCOLYSIS OF TRIOSE DERIVATIVES BY EXTRACTS OF TUMOUR AND OF MUSCLE[1]

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In previous papers [Scharles *et al.* 1935; 1, 2] it was shown that tumour extracts can produce considerable amounts of lactic acid from hexosephosphates. The behaviour of tumour extracts was found to differ from that of muscle extracts in several features, and it was concluded that "...carbohydrate breakdown in the two tissue extracts proceeds along different paths" [1935, 1]. Boyland & Boyland [1935], however, working also with hexosediphosphate arrived at the opposite conclusion, namely that "the rapid breakdown of hexosediphosphate by tumour extracts along the same paths as occur in muscle extracts gives support to the idea that the mechanisms of lactic acid formation in tumour and muscle are similar"; and they have maintained this position in subsequent publications [Boyland *et al.* 1937; Boyland & Boyland, 1938].

It is the purpose of this paper to report two types of experiment which demonstrate that glycolysis in muscle and tumour extracts follows different pathways.

Experimental

The experiments reported here consisted in a comparison of lactic acid formation by muscle and tumour extracts. As a preliminary step substrates were prepared, first, by the action of NaF-poisoned tissue extracts on glycogen in phosphate buffers. Secondly, hexosediphosphate and pyruvic acid were likewise used as substrates.

Methods. The methods and procedures used here, unless otherwise stated, were the same as those described previously [Scharles *et al.* 1935, 1].

Fluoride substrates. It has been known for many years that NaF prevents the production of lactic acid in muscle brei, although glycogen continues to disappear [Embden & Deuticke, 1934]. The carbohydrate which disappears is accounted for, in part at least, by the accumulation of phosphate esters as described by several authors [Embden & Zimmermann, 1924; Lohmann, 1930; Embden *et al.* 1933]. In such experiments these esters have been regarded as intermediates in the formation of lactic acid from glycogen. They are believed to accumulate as the result of a more or less specific inhibition of the reactions by which they normally produce lactic acid, with less or no inhibition of the reactions leading to their formation [Embden *et al.* 1933]. It was important, therefore, to determine whether any differences could be detected between tumour and muscle extracts in regard to the nature of the products formed

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under these experimental conditions. Accordingly, glycogen solution in phosphate buffer at *p*H 7·0 was incubated with muscle or tumour extract in the presence of NaF. After removal of the protein with trichloroacetic acid and of the inorganic P with Ba, the P esters were then isolated by precipitation of the Ba salts from alkaline alcoholic solution.

The data of Table I contain most of the experimental details of these experiments. The method of preparing the tissue extract, and other experimental and

Table I. *Preparation of NaF substrates*

				Phosphate			
Tissue	No.	NaF concentration *M*	Time of incubation hr.	Initial mg. P/100 ml.	Final mg. P/100 ml.	Esterified mg. P/100 ml.	Isolated mg. P/100 ml.
Rat muscle	M 4	0·005	4	398	287	111	41
,,	M 5	0·005	8	308	179	178	63
,,	M 6	0·005	4	102	62	40	21
,,	M 7	0·005	8	276	107	169	72
Mouse sarcoma 180	T 3	0·0075	8	124	52	72	35
,,	T 4	0·010	4	406	327	79	27
,,	T 5	0·0055	8	198	101	97	44
,,	T 6	0·005	8	327	210	117	44

analytical procedures were the same as those used previously [Scharles *et al.* 1935, 2]. The procedure for the isolation of the Ba salts was as follows.

The trichloroacetic acid filtrate (*c.* 5% CCl_3CO_2H) was neutralized in the cold with solid $Ba(OH)_2$, or a saturated solution and 2 g. $BaCl_2$ were added for each 100 ml. solution. The solution was then filtered to remove the precipitated $Ba_3(PO_4)_2$. The esters were isolated from the filtrate by the addition of 3 vol. alcohol. In some cases the esters were separated into two fractions, one precipitated at *c.* *p*H 7 and the other after making alkaline to thymol blue with NaOH. In other experiments all the esters were precipitated in one operation at about *p*H 8. Only 50% or less of the esterified P was recovered. The losses were found to be the result of inclusion of organic P in the $Ba_3(PO_4)_2$ precipitate, but the usual methods of recovering such losses by solution and re-precipitation did not greatly increase the yield. On the other hand, there was never any difficulty in recovering substantially all of the organic P of the $Ba_3(PO_4)_2$ filtrate. The precipitated esters were dried *in vacuo*, and dissolved in a little HCl. The Ba was removed with Na_2SO_4, and the resulting solution was neutralized to approximately *p*H 7. These solutions served as substrates for the action of muscle and tumour extracts, respectively. In Table II are given the data of a number of representative experiments of this type. In each case the lactic acid is expressed as m*M* per 100 ml. substrate solution, after subtracting the appropriate blanks determined for the substrate and enzyme preparations.

The data of Table II show that there is a considerable difference between the fates of the two types of substrate preparation. Muscle extract in every case produced more lactic acid from the muscle substrate preparations than did tumour extract. On the other hand muscle extract failed entirely to form lactic acid from the tumour substrate preparations. As a matter of fact in many instances a negative lactic acid value was found, indicating a disappearance of apparent lactic acid from the muscle extract when it was incubated with the tumour substrate preparation. Tumour extracts were always able to produce

Table II. *Lactic acid formation from NaF substrates**

Extract no.	Organic P mg./ml. incubation mixture	Lactic acid produced by	
		Muscle extract m*M*/100 ml.	Tumour extract m*M*/100 ml.
Muscle			
M 4A	1·76	0·43	0·10
M 4B	0·03	0·12	0·07
M 5A	0·76	0·35	0·25
M 5B	0·17	0·36	0·31
M 6A	1·31	0·47	0·01
M 6B	0·08	0·34	0·10
M 7	0·81	0·69	0·12
		Average 0·39	0·14
Tumour			
T 3A	1·18	−0·42	0·18
T 3B	0·05	0·06	0·30
T 4A	0·97	−0·13	0·27
T 4B	0·11	−0·10	0·42
T 5	0·76	0·01	0·33
T 6	1·12	−0·13	0·35
		Average −0·13	0·31

* I.e. substrates prepared from glycogen by the action of tissue extracts in the presence of NaF.

moderate amounts of lactic acid from these same substrates however. The conclusion seems inescapable that different substances are present in the two substrates, and that different enzymes are involved not only in their formation, but also in the glycolytic reactions.

Nature of the NaF substrates. Superficially the two substrates appear to be composed of much the same type of compound, as is shown by the data of Table III, and as yet we have been unable to isolate the substances responsible

Table III. *Analysis of NaF substrates*

Substrate no.	Phosphate			Reduction mg. glucose/ml.	$NaHSO_3$ titre ml. 0·005*N*
	Organic mg. P/ml.	Hydrolysable 30 min.	Hydrolysable 180 min.		
M 6A	2·62	8·3%	23%	8·6	2·71
6B	0·15	—	—	2·3	—
T 3A	1·18	10·6	23·5	5·8	1·64

for the differences in biological behaviour. There is a distinct possibility that some, at least, of these substances may belong outside the group of phosphate esters; because in several instances considerable glycolysis took place in solutions containing small amounts of P (Table II, M5B, T3B, T4B).

The data of Table III give the results of some analyses of three substrate preparations. The reducing values were determined by the procedure of Shaffer & Somogyi [1933]. The $NaHSO_3$ titre was obtained by adding excess $NaHSO_3$, oxidizing the excess with I_2, liberating the bound $NaHSO_3$ with $NaHCO_3$ and titrating with standard 0·005*N* I_2. The rates of hydrolysis of the phosphate esters were studied by estimating the amounts hydrolysed by *N* HCl after 30 and 180 min. in the boiling water bath. These analyses revealed no obvious differences between the two substrates. The chemical differences indicated by the difference in biological behaviour therefore remain to be demonstrated by fractionation and isolation procedures.

Glycolysis of hexosediphosphate. The glycolysis of hexosediphosphate was studied in some detail by Scharles *et al.* [1935; 1, 2] and by Boyland & Boyland [1935]. After the appearance of these publications, however, a paper was published by Meyerhof & Kiessling [1935–6] which shed new light on the glycolysis of this substance by muscle enzyme systems. In brief, it was found that triosephosphate, formed rapidly from hexosediphosphate in muscle enzyme systems, reacts vigorously with pyruvic acid with the simultaneous formation of phosphoglyceric and lactic acids. The phosphoglyceric acid was found to yield pyruvic acid with the concomitant esterification of glucose to hexosediphosphate. In this way a continuous production of lactic acid could ensue. The reaction involving triosephosphate and pyruvic acid is the key step in this scheme. Indeed, it was the first instance in which the velocity of lactic acid formation from supposed intermediates was found to be as rapid as that from glycogen itself. It was important, therefore, to determine whether tumour extracts are capable of catalysing this reaction.

Formation of triosephosphate. The splitting of hexosediphosphate into two molecules of triosephosphate in muscle extracts [Meyerhof & Lohmann, 1934], was found to occur also in tumour extracts [Boyland & Boyland, 1935]. We have been able to confirm this finding and to show that when tumour extracts are incubated with hexosediphosphate a considerable fraction of the organic P is maintained in the triosephosphate form. The results of one of five such experiments are shown in Table IV. 24 ml. Na hexosediphosphate solution containing 0·881 mg.

Table IV. *Fate of hexosediphosphate during glycolysis by tumour extract*

Time min.	Phosphate mg. P/ml. filtrate				
	Total	Inorganic	Triose-	Organic	Non-hydrolysable
0	0·731	0·106	0·009	0·625	0·087
1	0·737	0·111	0·059	0·626	0·087
5	0·731	0·117	0·119	0·614	0·086
140	0·743	0·261	0·149	0·476	0·072
200	0·758	0·305	0·150	0·453	0·060
960	0·780	0·653	0·016	0·127	0·046

P/ml. were incubated at 50° with 5 ml. tumour extract for the time indicated. The reaction was then interrupted by the addition of 4 ml. 40 % trichloroacetic acid. The solution was then cooled rapidly and analysed for total, inorganic and easily hydrolysable P, and for that hydrolysed in 180 min. at 100° in *N* HCl. In several instances the easily hydrolysable P was estimated after preliminary treatment with iodine and sodium carbonate. It was found to be unchanged in each instance, a finding which indicated the absence of glyceraldehyde phosphoric acid.

Effect of pyruvate. The rate of formation of lactic acid from hexosediphosphate was always increased markedly by the addition of pyruvate when muscle enzyme was used, but remained entirely unaffected when tumour enzyme was used. These facts are shown by the data in Table V which represent one of six similar experiments. The solutions were incubated 2 hr. at the temperature indicated. The pyruvic acid was freshly neutralized (with $NaHCO_3$ solution) and added in amounts equivalent, molecule for molecule, to the hexosediphosphate present.

The failure of tumour extracts to catalyse the reaction between triosephosphate and pyruvate indicates, we believe, a significant difference between the glycolytic processes of tumour and muscle systems. Whether or not this difference is due to the absence of the specific coenzyme which is necessary for this

Table V. *Glycolysis of hexosediphosphate in the presence of pyruvate*

Extract	Temp.	Hexose-diphosphate mg. P/ml.	Addition	Lactic acid formed m.*M*/100 ml.
Rat muscle	40°	0·42	—	0·62
		0·42	—	0·58
		0·42	Pyruvate	0·92
		0·42	Pyruvate	0·97
Mouse sarcoma 180	40°	0·34	—	0·53
		0·34	—	0·54
		0·34	Pyruvate	0·52
		0·34	Pyruvate	0·54
Mouse sarcoma 180	55°	0·44	—	0·87
		0·44	—	0·84
		0·44	Pyruvate	0·84
		0·44	Pyruvate	0·85

reaction in muscle [Meyerhof & Ohlmeyer, 1937] remains to be answered experimentally. That this is true is indicated by the finding of Boyland *et al.* [1937] that addition of yeast cozymase and adenylic acid to tumour extracts increases their glycolytic activity.

We have found that the rate of lactic acid formation is nearly directly proportional to the concentration of substrate and, within rather wide limits, independent of the amount of tissue extract added (Table VI, representative data from one of three experiments).

Table VI. *Rate of lactic acid formation as affected by the concentrations of enzyme and substrate*

Total vol. 4 ml. Incubated 3 hr. at 40°

No.	Sarcoma 180 extract ml.	Hexose-diphosphate mg. P/ml. mixture	Lactic acid formed m.*M*/100 ml.
1	2	0·44	0·96
2	2	0·22	0·54
3	2	0·11	0·34
4	1	0·44	1·02
5	0·5	0·44	0·91

There is a fundamental difference between the respective extracts used in the two laboratories. Boyland has shown that his aqueous extracts contain coenzyme-destroying enzymes which we find are absent from our saline extracts. This is suggested by the fact that the tumour adenylic acid deaminase can be extracted completely by water, but is absent or nearly absent from saline extracts made in this laboratory [Hitchings & Salter, unpublished]. In short, our experimental data and those of Boyland bear upon different aspects of the general problem.

Mawson [1936] recently has argued that the chief pathway of lactic acid formation by tumour from hexosediphosphate at 52° is via methylglyoxal and the glyoxalase system. This conclusion was based on the increased lactic acid which resulted from addition of glutathione to the extracts at 52°, although this did not occur at 38°. It was based, furthermore, on the fact that addition to the tumour extract of an acetone powder prepared from kidney decreased the yield of lactic acid. The latter observation was interpreted as the result of the anti-glyoxalase activity of the kidney preparation. It is capable however of a simpler

explanation: kidney extracts rapidly hydrolyse hexosediphosphate preparations without producing lactic acid. This is shown by the data of Table VII, which represent one of two similar experiments. A mixture containing 5·96 mg. P as

Table VII. *Action of kidney extracts on hexosediphosphate*

Time min.	Phosphate mg./ml. Total	Inorganic	Triose-	Organic	Lactic acid m*M*/100 ml.
0	0·650	0·124	0·006	0·526	—
10	0·650	0·320	0·144	0·330	—
21	0·661	0·462	0·082	0·199	0·01
30	0·666	0·534	0·051	0·132	—
45	0·661	0·595	0·025	0·066	−0·03

hexosediphosphate and 3 ml. 1 : 2 saline extract of mouse kidney tissue was incubated at 55°, total vol. 11 ml. At the stated intervals 2 ml. samples were withdrawn, added to 8 ml. 6% trichloroacetic acid, cooled immediately in an ice-salt bath, centrifuged and analysed. Parallel experiments were used for the estimation of lactic acid. It is obvious from these experiments that kidney preparations would reduce the lactic acid formation from hexosediphosphate by other tissue extracts, simply because of their phosphatase activity.

Discussion

The finding that tumour extracts fail to catalyse the reaction between triosephosphate and pyruvic acid which plays such an important part in the accepted scheme for muscle glycolysis [Meyerhof & Kiessling, 1935–6] is further confirmation of the opinion of Scharles *et al.* [1935; 1, 2] that glycolytic reactions in muscle and tumour follow different pathways at certain stages. This conclusion is confirmed also by the finding that different products are formed from glycogen by the two respective tissue extracts in the presence of phosphate and NaF. With respect to the pyruvic acid mechanism we are in agreement with Tsuzuki [1936] who found the mechanism for its formation and utilization (in conjunction with glycerophosphate) to be defective in at least three essential stages.

The glyoxalase mechanism [Mawson, 1936] of tumour glycolysis seems to us to be of doubtful importance as a physiological pathway, both because it is demonstrable only at unphysiological temperatures and also because its occurrence in such a wide variety of tissues [Mawson, 1937] indicates that it has little to do with the peculiarities of tumour glycolysis.

Conclusions

Differences in the pathways of muscle and tumour glycolysis can be demonstrated:

(1) in the properties of the products formed on incubating glycogen with the respective tissue extracts in the presence of phosphate and NaF, and

(2) in the failure of tumour extracts to catalyse the reaction between triosephosphate and pyruvate.

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